

TECHNICAL REPORT

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AD

INVESTIGATIONS ON FREEZING AND FREEZE-DRYING OF SELECTED FRUITS AND VEGETABLES

by

Basile J. Luyet

American Foundation for Biological Research
Madison, Wisconsin

Contract No. DA 19-129-AMC-81 (N)

July 1968

UNITED STATES ARMY
NATICK LABORATORIES
Natick, Massachusetts 01760



Food Laboratory
FL-74

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FOREWORD

This investigation was conducted on freezing and freeze-drying under some specific conditions on various selected fruits and vegetables.

Investigations were deemed necessary to obtain technical information as applicable to processing of various freeze-dried fruits and vegetables relative to projects under Combat Feeding Systems.

The work covered in this report was performed by the American Foundation for Biological Research, Madison, Wisconsin under Contract Number DA19-129-AMC-81(N). Dr. Basile J. Luyet was the Official Investigator and Dr. Alan P. MacKenzie was the Principal Associate Investigator.

The U. S. Army Natick Laboratories' Project Officer was Dr. Karl R. Johnson. The Alternate Project Officer was Dr. Edward E. Anderson.

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ABSTRACT

Investigations were made on the mode of invasion of fruit and vegetable tissue by ice and the changes produced by freezing and thawing and freeze-drying of the tissues as well as rehydration evaluations.

The diversity of the structure and composition of the fruits and vegetables investigated required the use of different methods of preparation and observation. In general three methods of exploring the effects of freezing and freeze-drying were used. (1) Direct observation of thin sections of fresh tissue under a cryomicroscope during freezing and thawing. (2) Observations of material fixed and stained after it had been freeze-dried. (3) Observations of material sectioned while in the frozen state or after freeze-drying.

Observations on the rehydration of thin tissue samples in a specially designed microscope demonstrated the dependence of the rehydration behavior on the structural features of the freeze-dried system.

INTRODUCTION

GENERAL PLAN AND DIVISION OF THE WORK

The overall plan in the research reported here was to study the effects of freezing and freeze-drying, under some specified conditions, on various fruits and vegetables, in particular: strawberry, peach, banana, pineapple, tomato, corn, pea and lettuce.

The processes of freezing and freeze-drying being so different by their nature called for different modes of approach; they will be treated in two separate parts of this report. Furthermore, a problem which soon gained predominance in the course of the study was that of the difficulties encountered in the freeze-drying of banana and of the reasons for such difficulties. The problem was attacked from different angles, and there resulted a considerable body of observations on the mechanism of freeze-drying and especially on the pathways for the elimination of water from the frozen material. These findings are a further justification for treating the mechanism of freeze-drying as a separate part (Part Two) of the entire project.

But the effects of freeze-drying — as distinct from its mechanism — remain to be treated in the part dealing with the effects of freezing (Part One).

In addition to the mechanism of freeze-drying and its effects, our program included a phase in the treatment of freeze-dried products, rehydration. This topic, together with the study of particular questions which arose in the course of the work, namely that of the behavior upon rehydration of compressed dry products, that of the effects of freezing in partially dehydrated tissues, and that of the effects of freezing and freeze-drying on fruits of different states of maturity will be the object of a third part of this report.

In each part we will consider (I) the problems and the general modes of approach, (II) the results obtained with the particular fruits and vegetables studied.

Finally, we will draw some general conclusions from the three parts.

PART ONE

EFFECTS OF FREEZING AND FREEZE-DRYING

I. PROBLEMS AND GENERAL MODES OF APPROACH

The main problems to be investigated were (A) that of the mode of invasion of the tissues by ice, and that of the changes produced by freezing-and-thawing and by freeze-drying on the tissues; (B) that of the amount of ice formed.

The main variables were to be (1) the type of tissue, (2) the treatments, in particular the freezing and freeze-drying rates, (3) the modes of observation and the ways of preparing the tissues for observation.

The diversity of structure and composition of the fruits and vegetables investigated called for the use of different methods of preparation and observation. Some tissues which have a firm consistency lent themselves well to ordinary histological techniques; others which have a high water content had to be mounted on firm supports; tissues with high sugar or starch contents called for particular methods of freeze-drying.

Preparation of Material and Observation. In general, we used three methods of exploring the effects of freezing and freeze-drying: (1) Direct observation of thin sections of fresh tissues, under the cryomicroscope, during and after freezing and thawing (the cryomicroscope used in this work was developed in our laboratory; see Luyet and Rapatz, 1957 and Rapatz and Luyet, 1957). (2) Observations of material fixed and stained after it had been freeze-dried. (Observations through the freeze-drying microscope during freeze-drying will be reported in Part Two.) In fact, we used various forms of these methods.

Freezing and Freezing Rates. Specimens were frozen either slowly in the air of a cold room at temperatures ranging generally from -2° to -20°C , or rapidly by immersion of thin layers into cooling baths at various low temperatures. When freezing did not occur spontaneously (at high, subzero temperatures), it was initiated by contact, at a point, with a precooled probe.

The course of cooling and freezing was recorded by means of a copper-constantan thermocouple, one junction of which was imbedded as nearly as possible in the center of the specimen. The couple was connected to an automatic recorder (Minneapolis-Honeywell "Elektronik"). As shown in the curves reproduced in Fig. 1, the temperature remained at the freezing plateau for a time of the order of a few seconds in the case of rapid freezing, and of one to a few hours in that of slow freezing.

II. RESULTS AND DISCUSSION

This section will be divided into two subsections corresponding to the two main problems mentioned: (A) the effects of freezing and freeze-drying, (B) the amount of ice formed. Each subsection will, in turn, be subdivided according to the kind of fruit or vegetable investigated, these being grouped together on the basis of the treatment they received.

(A) Observations on the Effects of Freezing and Freeze-Drying

(1) Direct Observations on Tissues of Tomato and Pineapple During and After Freezing and Thawing

1. TOMATO

Three series of experiments were run independently by different investigators on tomato; the first brought forth the principal events during freezing and the cytological changes caused by the freeze-thaw cycle; the second established more closely the relationship between temperature, cooling rates and structural changes and furnished more adequate photographic illustrations of the changes; the third was planned to supply information on the type and degree of injury by freezing at different rates.

1A. First Series of Experiments: Steps in the Freezing Process

Material. A ripe tomato, procured from a local grocer, was cut approximately in half with a serrated knife, along a plane perpendicular to the fruit axis, and then, with a razor blade, slices of parenchyma tissue, about 0.5 mm. thick, were cut along parallel planes.

Techniques. (a) Freezing in Alcohol Bath at -4° to -6° C. Each slice was sealed, with vaseline, between two plastic cover slips (No. 1, 22 mm.). The preparation thus obtained was attached to the "dropping mechanism" of the cryo-

microscope and abruptly lowered into an alcohol bath maintained at a constant temperature between -4° and -6° . The specimen, or a selected area of it, was first photographed before being frozen. Freezing was then initiated by "seeding" and the specimen was examined and photographed during the course of freezing. The flow of cold air was thereupon cut off, and the thawing of the specimen was studied as the alcohol bath gradually warmed up.

(b) F r e e z i n g i n A i r a t -20° C. The sealed preparation, resting on a microscope slide, was carried into a cold room at -20° and there placed on a chilled stereomicroscope through which the course of freezing was observed at a magnification of $80\times$. For the study of thawing, the slide bearing the frozen preparation was taken out of the cold room and mounted on a microscope of the same type at room temperature, and the course of thawing followed at the same magnification.

(c) F r e e z i n g w i t h D r y I c e. The sealed preparation was placed on a slide resting on the stage of the microscope, and frozen by means of a small chunk of dry ice, about 1 cm. on a side, laid on one corner of the cover slips. The course of freezing and subsequent thawing was followed under the low power objective.

Observations. We shall first outline briefly the salient features of the histology of the fresh, nonfrozen control materials, and then describe the sequence of events during freezing and thawing as observed under the microscope.

(a) H i s t o l o g y o f F r e s h , N o n f r o z e n C o n t r o l s. The parenchymal cells (Plate 1, Photo. 1) are spheroidal, ovoid or polyhedral, measuring from 400 to 500 μ across at their broadest points and from 100 to 200 - at their narrowest. They tend to be larger near the epidermis and become smaller and more rounded toward the center. They are loosely packed, the intercellular spaces being from 10 to 50 μ across. The nucleus (n in the photograph) is situated in the peripheral meshwork of

cytoplasm which invests the cell-sap vacuole. Immediately around the nucleus the cytoplasm forms a continuous sheet, from which strands radiate in various directions.

(b) C o u r s e o f F r e e z i n g. The course of freezing was essentially the same under the three sets of experimental conditions employed: application of dry ice, freezing in the air at -20° and freezing in alcohol at -4° to -6° . In each case three stages could be distinguished:

Superficial Freezing. Two fronts of flimsy, branched ice spears spread across the preparation independently of each other, one above and the other below the tissue, in the juice between it and the enclosing cover slips. These ice sheets are thin, flimsy and transparent, but recognizable by the somewhat reticulate appearance they impart to the cell surface (faintly discernible in cell A in Plate 1, Photo. 2).

Intercellular Freezing. Simultaneously with the passage of the superficial ice front, or shortly after it, ice penetrates between the cells and quickly fills the intercellular spaces. Dendritic fingers of ice then grow around the individual cells and gradually encase them, as is shown at d in cell A of Photos. 2-5. A slight shrinkage of the cell seems to accompany this encasement.

Intracellular Freezing. The cell interior then freezes suddenly and becomes intensely opaque. This sudden intracellular freezing occurs in random order in the several cells in the field of the microscope, as illustrated in the photographs of Plate 1, which were taken at 5-10 second intervals. Thus, cell C, transparent in Photo. 2, has become opaque in Photo. 3. The same change is noted in cell B (and also cell D) in Photos. 3 and 4, and in cell A in Photos. 5 and 6. Shortly after the sudden freezing, the ice recrystallizes and the cells lose some of their opacity (cf. Photos. 6 and 7). Recrystallization seems to commence in the circumnuclear area (Photo. 7).

(c) C o u r s e o f T h a w i n g. When the temperature is allowed to rise gradually, the ice around the nucleus, which had been the last to freeze, thaws first (Photos. 8 and 9). This is followed, in order, by the rest of the intracellular ice, the intercellular ice, and lastly the superficial ice. Thawing is completed between -1.5° and 0.5° .

(d) A p p e a r a n c e o f F r o z e n - T h a w e d C e l l s. The thawed cells show considerable alterations: (1) they appear slightly shrunken and more rounded in contour; (2) the cytoplasmic strands are torn and disorganized, and bits of them lie scattered throughout the cell; (3) the nucleus is displaced, probably as a result of floating during and after thawing (Photo. 10), since the cytoplasmic strands anchoring it have been severed.

1B. Second Series of Experiments "Flash Freezing" and Recrystallization

Technique. Thin slices (approximately $200\ \mu$ thick), mounted between microscope cover glasses in the dropping mechanism of the cryomicroscope, were frozen slowly by immersion in a cooling bath at -2° or at -5° , where they were "seeded". Similar preparations were frozen rapidly by immersion into baths precooled to temperatures ranging from -20° to -120°C . To thaw the specimens, we raised the temperature of the cryomicroscope bath at the rate of one degree per minute. In addition to the studies on slowly and rapidly frozen material, some observations were made on the effects of storage in the frozen state, at -5° or -10° , for various periods of time.

Observations. F r e e z i n g a n d R e c r y s t a l l i z a t i o n. In preparations frozen slowly at -2° only extracellular ice is formed; in preparations frozen at -5° intracellular "flash-freezing" takes place (darkening of some cells in Photo. 3 of Plate 2). At this temperature, both intra and extracellular recrystallization occur, as shown (1) by a decrease in the opacity

of the frozen cells (from Photo. 3 to Photo. 4), and (2) by the increase in size of the ice particles in Photo. 4, which was taken after a 10-minute storage in the frozen state at -5° . Similar results are obtained when the specimens are frozen at -10° .

In thin slices frozen rapidly at -120° , ice of rather fine grain is uniformly distributed throughout the preparation (Plate 3, Photo. 1). During slow rewarming, recrystallization begins at about -10° (coarser grain in Photo. 3), and becomes very pronounced at -5° and -3° (relatively large particles in Photos, 4 and 5). When the specimen is maintained at -3° , very large ice masses develop between adjacent cells (Photo. 6).

1C. Third Series of Experiments Degree of Injury

To test the conditions of the cells after freezing and thawing, we used two methods: (1) Observation of changes in the permeability of the membranes in cells exposed to a weak solution of potassium hydroxide after they had been stained vitally with neutral red; the cells which are permeable to KOH turn yellow (method described by Luyet, 1937). (2) Observation of changes in turgor when pieces of tissues are placed in distilled water.

Technique. (a) P e r m e a b i l i t y T e s t. Cells from unripe tomato were scraped and stained with neutral red for about two minutes. Then they were flooded with a 0.1% KOH solution. The excess solution was drained off and the cells were placed between glass coverslips of which the edges were then sealed with vaseline. The preparation was next immersed into the ~~alcohol~~ bath of the cryomicroscope maintained at -5°C . After about a minute, it was quickly lifted, seeded and reintroduced into the bath. Five minutes later the temperature of the bath was allowed to rise and the specimen melted.

(b) T u r g o r T e s t. Wedge-shaped pieces about 2.5 inches long and measuring some 0.3-inch on each side of the triangular cross

section of the wedge were cut from unripe tomatoes. Each piece contained epidermal and mesenchymatous tissues. One group of these slices was used as control; another was frozen at -5° ; a third was immersed in liquid nitrogen. Then, after thawing, they were all immersed in distilled water.

Observations. (a) C h a n g e s i n P e r m e a b i l i t y.

(1) Freezing at -5° results in change in color, that is, in an increase in permeability. (2) Freezing in liquid nitrogen does not produce an appreciable change, at least for a short time after thawing. (3) Superficial freezing does not affect cellular permeability. (4) Only the cells which have preserved their impermeability to KOH (that is, which do not turn yellow) do "flash freeze".

(b) C h a n g e s I n T u r g o r. The results are illustrated in Plate 4. Control, nonfrozen pieces (Photo. 1) acquire turgor and straightened out when placed in distilled water (Photo. 2). Pieces frozen at -5° (Photo. 4) or in liquid nitrogen (Photo. 6) keep the shape that they had before being frozen (Photos. 3 and 5, respectively).

Apparently freezing in liquid nitrogen has damaged the cells enough to permit the free passage of potassium hydroxide. Freezing at -5° caused a more severe injury, as is indicated by the permeability to KOH.

2. PINEAPPLE

Experiments similar to those described in the first series on tomato, under technique (a), were performed on pineapple.

Material. A ripe pineapple was cut across along a plane perpendicular to the leaf stalk, about one-third the way down from the attachment of the stalk, and a radial wedge of the fleshy tissue cut out. Then, with a thin stainless steel razor blade, slices 0.2-0.4 mm. thick were cut from the wedge and mounted for observation and experimentation.

Observations. (a) H i s t o l o g y o f N o n f r o z e n C o n t r o l s . The cells in the fleshy part of the pineapple are polyhedral, 250-300 μ long and 120-190 μ in the other two dimensions. They are rather closely packed, with considerable amounts of fiber between adjacent walls. The bulk of the cell interior is again occupied by a large, colorless vacuole. Surrounding the vacuole is a meshwork of cytoplasmic strands, except around the nucleus where the cytoplasm is more abundant and forms a continuous mass.

(b) C o u r s e o f F r e e z i n g . The mode of spreading of superficial ice and the random, sudden freezing of individual cells were essentially the same in the parenchyma of pineapple as in that of tomato. There was, however, no evidence of intercellular freezing and of ice encasement of individual cells prior to their sudden freezing. Further observations are needed to establish this point definitely.

In a second or third freezing of the same specimen, the superficial ice spread across the preparation in the same manner as in the first, but random, sudden freezing of individual cells did not occur. Instead, ice moved through the cells of the tissue in one continuous front.

(c) C o u r s e o f T h a w i n g . Just as in the case of tomato, the intracellular ice underwent recrystallization and disappeared first, and only when it was practically all gone did the superficial ice melt.

(2) Observations on Tissues of Lettuce, Strawberry and Corn, Freeze-Dried, Sectioned and Stained

Materials. — 1. Lettuce. The outermost 4 or 5 leaves from a head of lettuce were stripped off and from the flat, green portions of the leaf, squares, about 3/4-inch on a side, were cut and either frozen immediately or processed as controls. — 2. Strawberry. Fresh ripe strawberries of several varieties, in particular, that known as "Red Glow", were cut transversely into slices either 3/4 inch thick (for slow freezing) or 2 mm. thick (for rapid freezing).

— 3. Corn. Fresh isolated kernels of sweet corn (variety "Tender-most") were utilized either whole or after having been shaven off, along their broad, flat sides, to a final thickness of about 2 mm.

Techniques. F r e e z i n g. The specimens thus prepared were frozen either slowly in the air of a cold room at -5° or -20°C ., or rapidly in isopentane at -150° . In the first case (at -5°) freezing was initiated by contact, at a point, with a probe precooled in liquid nitrogen. In strawberry and corn, the course of cooling and freezing was recorded with thermocouples.

F r e e z e - D r y i n g. The specimens were vacuum-sublimed according to the following time-temperature schedule: (a) those frozen at -5° : for 72 hours at an ambient temperature of -10° ; (b) those frozen at -20° : for 72 hours at -20° ; (c) those frozen at -150° : for 7 days at -40° .

I n f i l t r a t i o n , E m b e d d i n g a n d S e c - t i o n i n g. The freeze-dried material was vacuum infiltrated with, and embedded in paraffin which had first been thoroughly degassed. It was then sectioned either 20 or 40-50 μ thick with a rotary microtome (thick sections being necessitated by the size of the cells).

S t a i n i n g. The sections were stained, according to the usual technique, with safranin and fast green dissolved in either alcohol or clove oil.

O b s e r v a t i o n a n d S t u d y. The stained sections were studied under the low and high powers of the microscope, representative areas being photographed through a Leitz "Micro-Ibso" attachment.

P r e p a r a t i o n o f N o n f r o z e n C o n t r o l s. All control specimens were fixed overnight in F.A.A. (formalin-acetic acid), then processed by the customary paraffin technique and stained with safranin and fast green.

P r e p a r a t i o n o f C o m m e r c i a l l y F r e e z e - D r i e d M a t e r i a l . In order to compare material freeze-dried according to our techniques with that commercially freeze-dried, we infiltrated the latter, whenever available, and processed it in the manner described for material freeze-dried in our laboratory.

Observations. The observations made on the three types of experimental material, namely lettuce, strawberry and corn, will be described and discussed separately.

1. LETTUCE

(a) C o n t r o l , N o n f r o z e n L e a v e s . The structure of the lettuce leaf (Plate 5, Photo. 1) conforms broadly to that encountered in leaves generally: a parenchymatous mesophyll is sandwiched between upper and lower epidermises. The mesophyll, some 9 to 11 cells deep, is not differentiated into the usual palisade and spongy tissue layers; rather the shape and arrangement of the thin-walled cells is pretty much the same throughout. The cells are polyhedral, more or less isodiametric, and rather compactly arranged. The overall water content for head lettuce is reported to be about 95%. (For reference, see Hayward, 1948, pp. 647-649.)

(b) L e a v e s F r o z e n a t -150° . There is no evidence of gross cell damage in specimens frozen rapidly at -150° and freeze-dried at -40° (Plate 5, Photo. 2): the epidermal and mesophyll layers show essentially the same structure as in the controls. Ice crystals that may be formed at this temperature are apparently too small to cause microscopically observable disruptive damage. Whether the ice is formed extracellularly or intracellularly, or both, cannot be ascertained from microscopic observations on our material.

(c) L e a v e s F r o z e n a t -20° . Specimens frozen at an intermediate velocity, that is, relatively slowly in air at -20° , show unmistakable signs of damage (Plate 5, Photo. 3). The epidermises contain cavities, the loci of ice masses which disrupted

2 or 3 adjacent cells. Much larger cavities, corresponding to 3-5 cells, occur in the mesophyll, especially just inside the epidermises and around the vascular bundles. These latter are generally surrounded by 2-4 such cavities. The ragged contours of the cavities and the fact that the surrounding cells show little or no evidence of shrinkage and compression would point to an intracellular rather than extracellular origin of the disruptive ice masses. The fluid-filled cell-sap vacuole of the mature plant cell might, moreover, be expected to serve as an ideal site for the initiation of crystallization.

(d) L e a v e s F r o z e n a t -5° . In material, frozen slowly at -5° , the mesophyll is completely disrupted by large, elongated ice cavities oriented more or less parallel to the leaf surfaces. Each cavity, seen in section, seems to have entailed the disruption of 3-8 cells in each of the 2-5 superimposed rows. In the tissue debris, compressed into narrow dark strips bordering the cavities, no intact cells and no nuclei or other cellular elements can be identified. The epidermal layers, too, are cavitated, badly mutilated, and broken in a number of places. Though the point of origin of the ice masses cannot be definitely ascertained, the ruptured epidermises point to an extracellular origin, as suggested by Asahina (1956) for ice formed in tissues at such high temperatures.

(e) C o m m e r c i a l l y F r e e z e - D r i e d H e a d o f L e t t u c e. As is to be expected when one freeze-dries an entire head of lettuce, and not just portions of isolated leaves, there is considerable variation in the size and distribution of the ice masses. The following general patterns of ice cavity distribution and tissue damage were noted:

(i) A pattern of injury roughly similar to that described for single leaves frozen at -5° or -20° , except that the orientation of the larger ice cavities is predominantly normal to the leaf surface (Plate 5, Photo. 4).

(ii) A compression by chains of ice masses, which formed centrally in the mesophyll, and squeezed the cells into two peripheral layers, each composed of 4-5 rows of cells. Within these compact masses of tissue the epidermal cells cannot be recognized as separate entities, though the nuclei and other cellular constituents are faintly discernible.

(iii) Chains of ice masses just internal to the epidermises which compressed the 5-9 rows of mesophyll cells into a narrow central strip between them (Plate 5, Photo. 5). Again, nuclei can be identified in the compressed cell strip.

The general pattern of ice cavity distribution in the commercially freeze-dried material suggests that, when an entire head of lettuce is freeze-dried: (1) freezing may often be initiated extracellularly; (2) centers of nucleation per unit area are fewer than when single leaves are frozen; (3) the ice masses grow slowly, resulting in (4) a dehydration and compression of the cells.

2. STRAWBERRY

(a) C o n t r o l , N o n f r o z e n F r u i t . The bulk of a strawberry, with a reported overall water content of about 90% consists of an enlarged "receptacle", to the periphery of which are attached the individual fruitlets or achenes. In the receptacle one can distinguish a centrally located pith and an outer cortex, rather sharply delimited by an inner ring of vascular bundles (cf. Havis, 1943). Portions of all three regions, i.e., pith p, cortex c, and vascular cylinder v.c., can be seen in Plate 5, Photo. 6, which represents part of a cross section of a mature strawberry. Pith and cortex both consist of large, thin-walled, very irregular parenchymatous cells. The dense vascular bundles are made up of small, thick-walled elements.

(b) S l i c e s F r o z e n a t -150° . As in the case of lettuce, so also here, the rapidly frozen specimens show no evidence of damage by compression or tearing (Plate 5, Photo. 7). The

nuclei, when visible, occur singly in "cavities", suggesting that each cavity corresponds to a single cell. Moreover, there is no unusual aggregation of cellular material along the middle lamellae.

(c) S l i c e s F r o z e n a t -20° o r -5° . Slices frozen slowly in the air at -20° or -5° again show considerable damage. Large, irregular, branched cavities occur in the parenchyma (Plate 5, Photo. 8, at c). The size of these cavities and the numbers of nuclei sometimes distinguishable along their borders suggest that each cavity involved the breakdown of 2-6 cells. The cellular material which had been compressed between the expanding ice masses can be seen aggregated along the middle lamellae.

Some ice seems to have formed also within the vascular tissue, as indicated by the larger clear areas scattered here and there within it.

In a few experiments the fruit slices were first suspended in strawberry juice (for 3-4 days), as is done in commercial canning. Just before being frozen in the air at -20° , they were removed from the juice and laid on a piece of filter paper for about 30 seconds to drain off the excess juice. This material sustained the greatest amount of damage: large ice cavities are scattered rather uniformly throughout the sections, their thick, dense, irregular borders consisting of the compressed cellular debris, in which nuclei and other cellular elements are no longer distinguishable. The ice masses seem even to have cut across and split the vascular bundles.

(d) C o m m e r c i a l l y F r e e z e - D r i e d S t r a w - b e r r i e s. The size range of the ice cavities in sections made of commercially freeze-dried strawberry slices is much wider than that encountered in the material frozen under laboratory conditions. The cavities seem to have resulted from the breakdown of 4 to 15 parenchyma cells (as opposed to 2-6 in the material frozen in our laboratory). There also appears to have been considerable damage to the vascular bundles.

3. CORN

(a) C o n t r o l , N o n f r o z e n K e r n e l s. In sections of corn kernels one distinguishes the following major regions: (1) pericarp (p in Plate 6, Photo. 2), made up of 5-8 layers; (2) endosperm (e in Photos. 1 and 2) consisting of an outer aleurone layer, a, 1-2 cells thick, and a much thicker starchy inner portion s; (3) germ (g in Photo. 1) which, in a complete longitudinal section, can be seen to consist of an embryo axis, a prominent cotyledon and a series of covering sheaths.

(b) K e r n e l s F r o z e n a t -150° . In general, there is a tendency for the germ to drop out of the frozen kernels during freeze-drying no matter what the freezing temperature is, probably due to differential shrinkage of the germ and the surrounding endosperm. Only kernels with the embryo still in position were analyzed.

The general appearance of kernels frozen rapidly at -150° (Plate 6, Photos. 3 and 4) is essentially the same as that of the nonfrozen controls (Photos. 1 and 2). There is no evidence of ice cavitation except, perhaps, in the pericarp. Cell inclusions, such as nucleus, starch and aleurone grains, are well preserved, and pericarp and aleurone layers remain in firm contact.

(c) K e r n e l s F r o z e n a t -5° o r -20° . Kernels frozen slowly at -5° or -20° show considerable damage. Within the germ large ice masses were formed, which caused a shrinkage and compression of the cells.

The contents of the large endosperm cells are also shrunken, and surrounded by irregular clear areas, the contours of which suggest that they were occupied by ice. There is, however, little evidence of cell rupture due to the spreading of the ice phase from cell to cell, as was noted in lettuce and strawberry material. This may be attributable to the lower overall water content of corn (reported as 76%, as compared to 90% and 95% for strawberry and lettuce, respectively).

The pericarp, besides containing ice cavities of variable size, is separated from the endosperm by a wide space, which may represent the site of a large ice sheet, but this is still not definitely established.

(d) C o m m e r c i a l l y F r e e z e - D r i e d C o r n K e r n e l s. In preparations of commercially freeze-dried corn kernels large ice cavities, each corresponding in size to 2 to 20 cells, are scattered throughout the sections. In addition to these there are smaller cavities within individual cells.

(3) Miscellaneous Observations by Various Methods on the Effects of Freezing and Freeze-Drying on Some Fruits and Seeds

This subdivision of the report furnishes additional information on material observed (a) after being sectioned in the frozen state, or (b) after being freeze-dried (but not otherwise treated, such as by staining), or (c) after freeze-substitution or (d) after treatment by a combination of procedures planned to permit a comparison of the effects of freezing and freeze-drying. The first two of these methods were used with peach, strawberry, pineapple, tomato, banana and pea, the third was used only in a special series of experiments on tomato, and the fourth only in a special series on corn.

Techniques. (a) P r e p a r a t i o n o f M a t e r i a l s f o r D i r e c t O b s e r v a t i o n i n t h e F r o z e n S t a t e. The specimens — slices 1 cm. or 1-2 mm. thick of strawberries and bananas, of the fleshy part of peach and pineapple, and of the interocular parenchyma of tomato; or entire peas — were frozen, the thicker specimens slowly in air at -20° , the thinner ones rapidly in isopentane at -150° . Then, in a -20° room, the frozen specimens were sectioned free-hand, as thin as possible, with a razor blade, and the sections, laid on a pre-chilled slide, were studied and photographed with a Reichert camera-microscope kept in the cold room.

(b) Preparation of Materials for Direct Observation After Freeze-Drying. The same fruits and seeds, frozen rapidly or slowly in the manner just described, were freeze-dried, the rapidly frozen specimens at an ambient temperature of -40° for 7 days, the slowly frozen ones at -20° for 72 hours.

The freeze-dried material was then prepared for microscopic study in one of two ways: (1) some of it was scraped off directly onto a microscope slide and examined under a stereoscopic microscope; (2) thin slices were cut from the outside of a specimen and their surfaces examined in the same manner as the scrapings.

(c) Preparation of Material for Observation After Freeze-Substitution or Freeze-Drying. Tomato slices, one mm. thick, and 2.5 cm. in diameter were suspended on a fine wire hook in a cold room at -20° and allowed to freeze slowly. To prevent supercooling, we placed a small ice crystal on the cut surface of the slice when the temperature had reached -1° . Similar preparations were frozen rapidly by immersion into isopentane baths at -150° . Ice was removed from both the rapidly and slowly frozen specimens either by "freeze-substitution" with ethanol at -78° or by freeze-drying at -40° . The material was then embedded in paraffin and sectioned with a microtome, and the sections were examined microscopically.

(d) Preparation of Material for Comparison of the Effects of Freezing and Freeze-Drying. The cells of the endosperm of commercially freeze-dried corn kernels show peculiar annular cavities between the cytoplasm and the cell wall (see Plate 9). To trace the origin of these cavities, we examined preparations of corn kernels which were sectioned while in the frozen state and others which were freeze-dried, embedded in paraffin and sectioned. In both cases we froze fresh corn kernels, in the "milky stage", in a cold room at -30°C . and sectioned them free-hand or with a microtome. To prevent the dehydration of the thin frozen sections we flooded them with cold

octane. — Kernels which were to serve for the determination of the degree of dehydration reached in the course of freeze-drying were cut into halves in the cold room, exposed to freeze-drying in a vacuum-desiccator at -30° , at a pressure of 0.3 mm. of mercury, weighed again and, after completion of the dehydration, embedded in paraffin, sectioned and examined.

Observations. (a) O b s e r v a t i o n s o n S e c t i o n s M a d e i n t h e F r o z e n S t a t e. Sections of the frozen fruits and vegetables studied in the frozen state have the appearance of a pile of spheres or of ellipsoids, each of which represents a single cell. The spatial relationship of one cell to another and the size of the intercellular spaces obviously depend on the manner in which the cells are packed together, and on their size and shape.

In both rapidly and slowly frozen material, the cells have smooth contours (e.g., Plate 7, Photos. 3 and 4), and, for a given kind of fruit, are fairly uniform in shape.

In sections of all six fruits and vegetables, either rapidly or slowly frozen, the gross tissue structure appeared undisturbed: there was no evidence of tearing, compression or distortion, and no large ice masses could be found between the cells. Each cell appeared to be either a solid block of ice (large white areas in Photo. 2 of Plate 7) honeycombed by a meshwork of unfrozen material, or to be filled with a few to many particles of ice (Photo. 1 of the same plate).

In rapidly frozen tissue the particles of ice are very small and form a fine meshwork which, by scattering the light, renders the cells somewhat opaque (Plate 7, Photos. 1, 3 and 5, to be compared, respectively, with Photos. 2, 4 and 6). In slowly frozen tissue, the ice particles are fewer and larger, and the frozen cells are more transparent (Plate 7, Photos. 2, 4 and 6).

Sections of slowly frozen fruits could be readily photographed, even if they were two or three cells thick, while it was usually difficult to photograph even a single cell of a rapidly frozen fruit;

sections two or three cells thick of such a fruit were completely opaque.

(b) O b s e r v a t i o n s o n S c r a p i n g s f r o m F r e e z e - D r i e d P e a c h a n d P e a . The cell walls in rapidly frozen specimens are sculptured with many fine wrinkles which strongly scatter the light and cause the walls to appear opaque.

In scrapings from slowly frozen specimens the cell walls are fairly transparent and do not show many wrinkles; wrinkles that do occur tend, however, to be coarser and deeper than in rapidly frozen material.

(c) O b s e r v a t i o n s o n T h i n S e c t i o n s o f F r e e z e - D r i e d P e a c h , S t r a w b e r r y a n d P e a . The cells of both the rapidly and the slowly frozen material appear to be intact, and there is no evidence of large intercellular ice cavities.

The walls of the slowly frozen specimens again appear transparent and relatively smooth; whereas those of the rapidly frozen ones are opaque, with a fine network imprinted on them.

(d) O b s e r v a t i o n s o n S e c t i o n s o f F r e e z e - S u b s t i t u t e d o r F r e e z e - D r i e d T o m a t o . The paraffin-embedded pieces of tomato being cut, as was already mentioned, into 10-micra thick sections, the amount of cytoplasm remaining in the cells was barely sufficient to be observable; one sees only traces of it in the photographs reproduced in Plate 8; but the cell-wall framework is very conspicuous.

In the rapidly frozen specimens (Plate 8, Photo. 2), the cells are distinct units, similar in size and shape to those of the controls (compare Photo. 2 with Photo. 1). The cell walls, however, appear to be broken and shredded. This same observation was made in both freeze-dried and freeze-substituted specimens which, while not ruling out the possibility of damage resulting from processing the material, suggests that these breaks may be the result of freezing.

In the slowly frozen specimens (Photo. 3) the individual cells are no longer recognizable; what remains is a framework apparently resulting from an aggregation of the walls and contents of adjacent cells; the meshes of the framework were probably occupied by large blocks of ice (now empty cavities).

(e) O b s e r v a t i o n s o n S e c t i o n s o f F r o - z e n a n d F r e e z e - D r i e d C o r n . As shown in Photo. 1 of Plate 9, the cells of the endosperm of commercially freeze-dried corn contain, in addition to numerous small cavities within the cytoplasm, large annular cavities between the cytoplasm and the cell walls. The same structures were observed in material freeze-dried in our laboratory, and also, as shown in Photo. 2 of Plate 9, in material sectioned in the frozen state. It is, therefore, evident that the bulk of the annular cavities was formed in the course of freezing.

Determinations of the degree of drying attained indicated a loss of 48% in the weight in 5 days, which represents a still quite limited dehydration. Since, in that work, we obtained no information on the onset of secondary freeze-drying, the problem still remains of the extent to which the large annular cavities and the small intraprotoplasmic cavities are attributable to the shrinking resulting from the removal of nonfrozen water (pseudo freeze-drying).

(B) Determination of the Amounts
of Ice Formed in Some Fruits and Vegetables
Frozen to Various Temperatures from -2° to $-30^{\circ}\text{C}.$

The amounts of ice formed at the temperatures indicated were determined calorimetrically according to the following procedure:

Apparatus and Method. A Dewar flask of 260 ml. capacity served as calorimeter. It was placed in a Styrofoam-insulated enclosure in which an ice-water bath maintained a constant temperature. A thin plastic rod extending through the insulation served to operate a small, electrically driven, propeller-type stirrer. An opening through the

Styrofoam lid provided access to the calorimeter. The calorimetric fluid consisted of 100 grams of distilled water; its temperature was measured by means of a copper-constantan thermopile connected to a millivolt recorder through a DC amplifier.

The water equivalent of the calorimeter and stirrer was determined by the standard method of making a run with a known weight of ice at a given temperature. — A platinum resistance thermometer was used to calibrate the thermopile and recording system. The 11-inch span on the recorder covered a temperature range of about 3.3 degrees, from 5.1° to 8.4°C.

Pieces weighing 3 to 5 gms., cut from the fruit or vegetable to be tested, were wrapped in aluminum foil, weighed, and frozen in cold air at the desired temperature. Then, after removal of the wrapping, they were dipped into the calorimeter and the resulting change in temperature was measured. — The pieces consisted of cylinders cut across the fruit in the case of banana and strawberry, wedge-shaped sectors of such cylinders in the case of peach, tomato and pineapple, and discs cut from the leaves in the case of lettuce. (Only the flesh of the bananas was included.)

The amount of ice formed was calculated from the drop in temperature of the calorimeter fluid according to the method routinely used in calorimetry. The specific heats of the frozen and unfrozen materials were obtained from the Refrigeration Data Book (1952).

Experimental Data and Comments. The amounts of ice formed when the specimens were frozen at -2°, -5°, -10°, -15°, -20° and -30° are given in Table 1; the corresponding data on five fruits and vegetables on which we have sufficiently complete information are plotted in Figs. 2, 3 and 4, in the form of "composition freezing curves" (in Luyet's terminology, 1966), that is, in curves which give the amounts of solids present (S), of ice formed (I) and of water remaining unfrozen (W) at the particular temperatures investigated.

A comparison of the data of Table 1 leads to the following conclusions or suggestions:

(a) The amounts of ice formed a few degrees below the freezing point (in the range of -2° to -5°) represent generally some three-fourths or more of the water freezable at any temperature. The amounts formed at -20° seem to approach an asymptotic value.

(b) In general, the fraction of the total water content not frozen when the curve is nearly asymptotic varies in the same direction as the solid contents. This means simply that some water is prevented from freezing by the presence of solids; in a sense, that water is bound to the solids in such a way that it is not available to form ice. The extreme cases are, on the one hand, banana (Fig. 4) which has the lowest water content and, on the other, lettuce and tomato (Fig. 3) which have the highest.

The ratio of the amount of water (W) remaining unfrozen at -20° to the total solids (S) is approximately the same, varying from 0.50 to 0.63 for tomato, pineapple, peach, strawberry and banana; it jumps to 1.2 for lettuce.

(c) Another aspect of the relationship between nonfrozen water and solids is the water content of the nonfrozen fraction, that is, the quantity represented by the ratio $w/(W+S)$. It varies from about 33 to 39% in the case of tomato, pineapple, peach, strawberry and banana, and jumps to about 55% for lettuce.

As they are, these figures can only give a hint on the general trend in water binding, and they are silent on solute concentration. To have really significant information one should establish the water binding coefficient and solubility of the substances present. But this would constitute an extensive research project by itself.

TABLE 1

Amounts of Ice Formed in Various Fruits and
Vegetables Frozen at Various Temperatures from -2° to -30°C.

(The number of figures listed at a given temperature, for a given fruit or vegetable, indicates the number of determinations made.)

Material	Percent of Sample Frozen (wt./wt.)					
	-2°	-5°	-10°	-15°	-20°	-30°
PEACH	47.8	66.0	77.2		78.6	
	49.8	70.9	77.8		79.9	
	51.7	70.5	75.6		79.5	
					80.4	
Average:	49.8	69.1	76.9		79.6	
PINEAPPLE	38.8	54.6	66.0	70.0	77.8	78.2
	39.4	55.0	66.7	72.0	78.0	79.8
	40.2			73.6		
	40.4					
Average:	39.7	54.8	66.4	71.9	77.4	79.0
LETTUCE	79.9	88.1	91.5		93.1	91.4
	78.7	88.1	92.0		93.3	93.1
	76.6	89.6	92.8		93.7	
Average:	78.4	88.6	93.1		93.4	92.3
TOMATO	73.2	85.2	88.7		93.0	93.3
	76.0	86.0	89.0		93.1	93.7
	70.8		89.6		93.4	
					94.4	
Average:	73.3	85.6	89.1		93.6	93.5
BANANA		38.4	51.5		62.1, 64.7	66.1
		39.2	52.4		61.9, 62.4	67.1
		44.4	53.2		62.1, 63.9	67.3
					63.1, 63.7	
Average:		41.7	52.3		63.0	66.8
STRAWBERRY					80.9	
					84.5	
					85.6	
Average:					83.7	

PART TWO

MECHANISM OF FREEZE-DRYING

1. PROBLEMS AND MODES OF APPROACH

As has already been stated, the study of the mechanism of freeze-drying was introduced into this program because of the difficulties encountered in attempts at freeze-drying bananas. The entire work is therefore centered on banana and its components, and the search is basically for the reasons for the difficulties, that is, for the resistances offered by that fruit to the sublimation of the ice and the evacuation of the water vapor resulting from sublimation. The main problems which we investigated were, therefore: (1) those concerned with the factors that control the rate of dehydration — some such factors are internal: the structure of the tissues, their chemical composition, the pattern of ice distribution; others are external: the temperature and rate of freezing and the temperature of sublimation — (2) the mechanisms by which the water molecules are liberated from the frozen complex and are evacuated through the labyrinthic systems of pathways.

Our first task was to make gross determinations of the rate of dehydration of frozen banana tissue and of some of its components, in particular, banana juice and sugar solutions, under some particular conditions of freezing rates and drying temperatures. The findings in that work are presented in Section (A) hereafter. Then we studied the mechanism of freeze-drying by direct observation through the "freeze-drying microscope" of the progress of the "drying front", and by attempts at tracing the pathways available to escaping water vapor (Section B). Next (Section C) we recorded freeze-drying curves with a highly sensitive microbalance-assembly designed to furnish information on the stages in the process of freeze-drying. Finally (Section D) we obtained additional information on the pathways by studying under the electron microscope some details of the ultra-structure of frozen material which are of significance in the elucidation of the freeze-drying mechanism. (The "freeze-drying microscope"

and the "freeze-drying microbalance assembly" have both been developed in our laboratories: they are described, respectively, in papers by MacKenzie, 1964 and by MacKenzie and Luyet, 1964b.)

II. RESULTS AND DISCUSSION

(A) Gross Measurements of Freeze-Drying Rates in Banana and Its Components

To obtain information on the effects on the freeze-drying rate (1) of temperatures (when sublimation is limited to a given area), (2) of the histological structure and (3) of the composition of the material, we divided the work into three parts: (1) Freeze-drying from one face of a banana slice, as a function of the ambient temperature. (2) Comparative freeze-drying rates of central and peripheral tissues of banana, and (3) Comparative freeze-drying rates of whole banana slices and of some of their constituents.

(1) Freeze-Drying from One Face of a Banana Slice, as a Function of the Ambient Temperature

Technique. A slice, 2 cm. thick, and weighing 10 g., cut from near the middle of a ripe banana (where it had a diameter of slightly more than 2.5 cm.) was pushed gently into a 2 cm. length of thin-walled nylon tubing 2.5 cm. in internal diameter. The cylindrical specimen, thus encased, was set, on end, on the aluminum base plate of a freeze-drying vessel, and frozen in air in a cold room at -30°C .

The vessel, containing the frozen specimen (the latter being free to dry from its upper circular face only) was attached to a freeze-drying apparatus which offered a very low resistance to vapor flow, and which permitted the periodic determination of the amount of water vapor removed (MacKenzie and Luyet, 1964a).

During freeze-drying, the specimen chamber was immersed in an alcohol bath maintained either continuously at -15° or successively

at -30° , -25° , -20° and -15° , and the amount of water removed was measured every 10 minutes.

Factual Data and Their Significance. The rate of water loss at a constant ambient temperature of -15° , as shown in Curve A of Graph 1, Fig. 5, was practically constant during the first 1 hour and 40 minutes, during which time the specimen lost 1.4 grams of water. For the 10-gram specimen used, with an initial water content of about 75%, the rate of freeze-drying was constant till about 1/5 of the water had been removed.

In Curve B of the same graph are shown the rates of sublimation of another specimen exposed successively to different ambient temperatures. The graph consists of a series of straight-line segments, one for each temperature used. Thus, under these conditions, too, the drying rate was constant for a given ambient temperature.

From the slopes of segments a, b, c and d of Curve B, the rates of freeze-drying at -30° , -25° , -20° and -15° are found to be in the ratio of 1 : 1.5 : 2.5 : 3.7. The ratios of the vapor pressures of ice at these same temperatures, as given in the "Handbook of Physics and Chemistry", turn out to be 1 : 1.7 : 2.7 : 4.3. The close parallelism between the ratios of the vapor pressures and of the rates of water loss leaves little doubt that the vapor pressure of the ice provides the driving force in the freeze-drying of banana. Furthermore, since the rate of water loss is in direct proportion to this driving force, it would appear that the peculiar resistance of the tissue to freeze-drying is of a type which does not vary with temperature in the range -15° to -30° .

A comparison of the slope of Curve A (of a specimen dried continuously at -15°) with that of segment d of Curve B shows that the rate of drying at -15° is the same irrespective of the stage in the drying process. It would, therefore, appear safe to conclude that, if freeze-drying were to be conducted continuously at -30° , -25° or -20° , the first 20% of the total water content would, in each case,

be lost at a constant rate, and that the rates would be of the same order as those found for segments a, b, and c of Curve B.

The sublimation rates experimentally established for banana slices are some 10 to 100 times lower than those theoretically expected on the basis of calculations for a system in which open channels connect the interior of the sample with the surface and thus permit a direct flow of water vapor through the freeze-drying tissue. The very low measured freeze-drying rates lead one to conclude that barriers of some kind exist in the tissue.

In view of the constant rate of sublimation for a considerable time a considerable water loss, it would appear that the barrier to vapor flow is of a constant nature, and that the hindrance offered by a progressively thicker superficial layer of freeze-dried tissue is comparatively negligible. The major and constant barrier must, accordingly, be sought for at or near the surface from which the freeze-drying occurs.

The specific nature of this barrier has not yet been established. It may, however, be suggested that, in the cutting of the banana slice, the sugary, starchy, proteinaceous contents of the ruptured cells become smeared over the cut surface and partially seal or occlude the channels or pores through which the water vapor must escape.

(2) Comparative Freeze-drying Rates of Central and Peripheral Tissues of Banana

From the results recorded in the previous section it would appear that differences in histological structure exert a minor effect, if any, on the velocity of freeze-drying, at least during the early stages of the process. In order to check this point further, we decided to compare the rate of freeze-drying of the central core and of the peripheral tissues of banana.

Technique. A cylindrical disk and a peripheral ring were cut, by means of a cork borer, from a slice of banana 1 cm. thick, and a

similar disk and ring from a slice 1 mm. thick. The size of the borer was such (about 13 mm.) that the surface/volume ratios for core and annulus were approximately equal. The 1-mm. thick specimens were frozen rapidly in isopentane at -150° , the 1 cm. thick specimens slowly in air at -30° . All the frozen pieces were freeze-dried individually in specimen chambers maintained at -10° , the water loss being determined every 10 minutes.

Factual Data and Their Significance. The results are shown in Graph 2. In both the rapidly and the slowly frozen specimens (Curves A_1 , A_2 and B_1 , B_2 , respectively), the peripheral annulus (A_1 and B_1) dried somewhat more rapidly than the central core (A_2 and B_2), the divergence between the two being greater in the rapidly (A_1 , A_2) than in the slowly (B_1 , B_2) frozen material.

Another interesting point to note is that core and annulus frozen rapidly (A_1 , A_2) freeze-dry considerably more rapidly than the core and annulus frozen slowly (B_1 , B_2). The difference can probably be attributed to the smaller size and correspondingly greater surface to volume ratio of the ice particles formed in rapid freezing.

(3) Comparative Freeze-Drying Rates of Whole Banana Slices and of Some of Their Constituents

Technique. A 10-gram slice of banana, such as that used in the experiments described in Section (1) above, and 10-gram samples of: (1) banana paste, (2) banana juice (obtained by straining banana juice through cheesecloth) and (3) a sugar solution comparable in composition and concentration to banana juice (10% sucrose and 8% dextrose by weight), were each enclosed in short lengths of thin-walled nylon tubing having an internal diameter of 2.5 cm. All the samples, of identical size and shape, were frozen in the air at -30° in the manner described under (1) above; in the case of the sugar solution an additional sample was frozen in alcohol at -78° . The frozen

specimens, which had only one face — the upper circular one — exposed for ice sublimation, were freeze-dried at an ambient temperature of -30° . The amount of water lost during the freeze-drying was measured at 10-minute intervals.

Factual Data and Their Significance. The rates of freeze-drying for the different samples are plotted together in Fig. 5, Graph 3. It is seen that of the four different samples treated under identical conditions, the unstrained paste (Curve A) freeze-dries most rapidly, the strained paste or juice (Curve E) least rapidly, and the intact slice (Curve C) at a rate intermediate between the two. The more rapid freeze-drying of the unstrained than of strained banana paste leads one to suspect that the presence of cellulose, especially in the form of free fibers, enhances freeze-drying. The organization of the fibers in the tissue architecture would not destroy their efficacy, but would reduce it.

Closely paralleling the low rate of freeze-drying of banana juice is that of the sugar solution (Curve D), especially at the beginning of the process. This is doubtless due to the tendency of sugar solutions to form a glass when frozen. (Salt solutions of corresponding concentrations freeze-dry rapidly, for the probable reason that the salts crystallize out during freezing.)

It should also be noted that the rate of freeze-drying of the sugar solution depends on the rate at which the solution was frozen: the specimen frozen at -78° (Curve B) dried considerably more rapidly than that frozen at -30° (Curve D). Unfortunately the ice in the frozen sugar solutions recrystallized quite rapidly at -30° , the temperature at which the freeze-drying experiments were conducted. Thus we hesitate to give much importance to the quantitative analysis of the freeze-drying behavior of these solutions. The frozen banana and the frozen paste and juice apparently did not recrystallize to an appreciable extent at -30° ; thus the comparison of their freeze-drying rates is probably valid.

(B) Study with the "Freeze-Drying Microscope"
of the Mechanism of Freeze-Drying in Banana and Its Components

Since this section of the report consists primarily in the study of a state of instability undergone by frozen material during its dehydration (freeze-drying), we shall first describe the fundamental phenomenon involved. MacKenzie (1964, 1965) discovered that one of the greatest changes in the mechanism of freeze-drying encountered in solutions takes place at a temperature which has been the subject of investigations for 25 years in Luyet's laboratories. This is the temperature at which a solution, cooled rapidly enough to remain transparent, turns opaque during rewarming. Luyet and Rapatz (1958), on the basis of their observations (1957) that the transparent material had generally crystallized into spherulites, considered the phenomenon as a recrystallization and accepted for the temperature at which it occurs the designation "recrystallization temperature". Such a temperature is determined primarily by the nature of the solute and is only slightly affected by the concentration of the solution. Luyet (1960 and 1965) give tables of the values obtained for a certain number of solutes. The value reported for sucrose (as established by Luyet in 1939) is -31.4° for a 1M and -31.8° for a 2M concentration. As we shall see, this is about the temperature at which banana juice and banana tissue lose their stability.

When a rapidly frozen solution is rewarmed its instability is manifested by a recrystallization; when it is freeze-dried at gradually rising temperatures, its instability is manifested by the "collapse" of a structure which is "retained" at lower temperatures. This study of collapse and retention was carried out with banana juice (often called hereafter banana "supernatant" after the mode of preparation used, which included a centrifugation) and with thin slices of banana.

Technique. In the case of banana supernatant fluid, thin layers were placed between a cover slip and one window of the freeze-drying

chamber of the microscope assembly and frozen either by controlled cooling of the assembly, or by immersion of the chamber in a cooling bath and subsequent transfer of the chamber to the microscope. (For a description of the freeze-drying microscope assembly we refer the reader to the original paper by MacKenzie, 1964.)

In the case of pieces of banana, thin layers were cut from a frozen fruit in a cold room at -25° and mounted firmly between a cover slip and one window of the freeze-drying chamber. The assembled chamber was then carried from the cold room to the pre-cooled microscope to which it was attached.

Freeze-drying was carried out at various temperatures, controlled to ± 0.1 degree, in the range -20° to -50°C . and at a pressure of less than 5 microns of mercury at the condenser. Changes in the temperature of the specimen in the course of freeze-drying were obtained by controlled variations of the temperature of the cooling bath incorporated into the microscope assembly.

One may remark that, with the arrangement described, the gradient in water vapor pressure in the dry matrix varied from saturation (with respect to ice) at the subliming interface to a very low value (about 1% relative humidity) at the edge of the sample.

Observations. (a) B a n a n a S u p e r n a t a n t. Banana supernatant behaved in a very similar way to sucrose solutions and to solutions of many other non-crystallizing solutes studied in our laboratory during the past few years: they all exhibited the same "collapse phenomenon" above a certain freeze-drying temperature, T_c (the "collapse temperature"), and a softening, on warming, of those parts of the matrix produced by freeze-drying at temperatures below T_c .

The collapse phenomenon in banana supernatant is illustrated in the four photographs of Plate 10. Freeze-drying at -36° (Photos. 1 and 2) and at all other temperatures lower than -31° caused the formation of a solute matrix, free of ice, which has the same microscopic

appearance as the material in the original frozen state. In such cases, we will say that freeze-drying progresses with "retention" of structure. Freeze-drying at -26° (Photo. 3) and at all other temperatures higher than -31° , up to the melting point of the system (about -2°) caused the solute in the subliming interface to undergo viscous flow with consequent rearrangement of the material comprising the solute phase distributed originally between the ice crystals. In those cases, we will say that freeze-drying takes place with simultaneous "collapse" of the solute matrix (MacKenzie, 1965).

As was observed with other solutions, the capability of collapse was preserved when a preparation exposed for a time to temperatures above T_c was cooled again below T_c ; such a preparation did collapse upon being freeze-dried above T_c . Thus, as shown in Photo. 3 of Plate 10, a rise in freeze-drying temperature resulted in collapse, a subsequent drop in temperature caused collapse to cease (Photo. 4), but a second rise caused a resumption of the collapse phenomenon. This could be repeated several times; collapse behavior was always encountered on warming, and suppressed on cooling, each time at the same temperature. There was no evidence of hysteresis. Judged by visual standards, the range over which the change in mechanism occurs covered only two or three degrees C.

Preparations of banana supernatant also showed a post sublimation softening, on warming, as did many other solutions examined previously; that is, the solute matrix resulting from freeze-drying at temperatures below T_c softened and collapsed when the temperature was raised above T_c . The softening occurred to a lesser extent in the areas further removed from the position of the subliming interface at the time the freeze-drying temperature was raised (that is, in the areas nearer to the outside edge of the sample). Seen in the freeze-drying microscope, this softening phenomenon had the appearance of a melting of the empty matrix, proceeding in the same direction as the flow of water vapor. The softening progressed to a greater extent

when the final freeze-drying temperature was higher; when the temperature was raised above -31° the sample appeared to collapse in both directions from the "front", that is, toward the outside edge (within limits) and toward the interior of the sample. This latter collapse process continued as long as the freeze-drying temperature was maintained higher than -31° .

(b) B a n a n a S l i c e s. Specimens from three kinds of bananas were examined: (1) from ripe bananas, (2) from yellow-green bananas, both frozen rapidly (1 cm. thick slices immersed in liquid nitrogen), (3) from ripe bananas, frozen slowly (same-size slices placed in cold room at -30°).

Almost the same collapse behavior was observed in the very thin slices (about $15\ \mu$ thick) of the various banana samples as in the frozen layers of the supernatant fluid. The collapse temperature, however, was found to be -25° with rapidly frozen samples (both ripe and unripe) and -21° with slowly frozen ones (ripe).

The thin slices, furthermore, contained considerable debris in the form of starch grains and cell-wall fragments (the sections were much thinner than the distance across the cells). Collapse caused this debris to be swept into aggregates, except where it already adhered to the cover slip and did not move. The resultant pattern was that of disorganized and clumped aggregates instead of fields of uniform appearance. Thicker samples, in which entire cells would have been present, could not be examined in detail since they did not transmit sufficient light either in the frozen or in the freeze-dried states.

When the samples were freeze-dried below their respective collapse temperatures, the starch grains and cell-wall fragments were seen to remain in place, held by the rigid matrix of the cell fluid solids, as one might have expected.

Comments. Studies of a number of frozen solutions have shown that the collapse temperature T_c coincides, in almost every case, with T_r , the recrystallization temperature, that is, with the temperature below which concentrated, rapidly frozen solutions do not exhibit recrystallization upon rewarming. Such an observation suggests that the collapse and recrystallization processes, although occurring as the result of different driving forces, encounter resistances which are similarly temperature-dependent. One naturally thinks of the viscosity of the solute in the regions just freed of ice as the source of the resistance; that viscosity may increase abruptly to high values when the temperature is lowered by one or two degrees below T_c ; upon warming, the tendency to collapse would increase as the viscosity decreases. (Undoubtedly it is the surface tension of the interface between the concentrated solute and the vacuum, tending always to reduce the area of that interface, which constitutes the driving force for the collapse phenomenon.)

If the foregoing observations hold true for banana supernatant, we can place the temperature below which recrystallization of the ice will not occur in frozen supernatant as -31°C .

In other experiments we found that collapse temperatures for solutions of several substances were generally intermediate between those for the pure components, except where large quantities of salts were present. The value of -31° for supernatant may be explained largely in terms of the swamping quantity of sucrose present. Fructose (T_c of -48°) and the gums and proteins (T_c 's about -10°) evidently balance one another in their effects.

The postsublimation softening resulting from rises in the freeze-drying temperature is probably attributable also to some discontinuous change in molecular mobility, itself dependent on the extent of desorption drying of the matrix.

(C) Study by Microbalance-Recording
of the Mechanisms of Freeze-Drying
in Sucrose Solutions and Banana Juice

Apparatus and Technique. The apparatus used in this study was designed to permit the continuous recording of weight changes and, therefore, of sublimation velocities, in small samples freeze-dried under predetermined conditions. The system incorporates a Cahn RG Electrobalance used in combination with a thermocouple, so as to allow the simultaneous recording of both sample weight and sample temperature. (For further details we refer the reader to the original paper by MacKenzie and Luyet, 1964b.)

Sample containers were made from two square pieces of aluminum foil measuring 2 cm. on edge, separated by a 1.2 mm. thick polypropylene ring cut open on one side, the pieces being held together by special forceps. A 1-mil copper-constantan thermocouple was located at the geometric center of the container, its leads being attached to the polypropylene ring with minute quantities of rubber cement. The solution of sucrose to be tested was then placed in the container and frozen by abrupt immersion in isopentane at -140°C . The preparation was drained of isopentane on crushed dry ice and transferred to a room at -45° where the foil was removed and the specimen placed in the glass vessel in which it was to hang by its thermocouple leads from the beam of the freeze-drying microbalance.

Experimental Data. In this investigation we recorded some 25 freeze-drying curves of which we select 13 that seem fairly representative of the essential features of the process. Of these 13 curves, 4 were obtained with 25% sucrose, 5 with 36% sucrose and 4 with banana juice; these three sets of curves are plotted, respectively in Figs. 6, 7 and 8. We shall now examine and analyze these curves.

(a) S u c r o s e S o l u t i o n s o f 2 5 % C o n c e n -
t r a t i o n (Fig. 6). (1) The first striking feature in the curves of Fig. 6 is the great difference in dehydration rate when drying is done at -25° and when it is done at the three other temperatures. Whereas

there are only slight differences in rate for the freeze-drying temperature varying from -50° and -36° , there is, at all values of the ordinate, that is, at all depths of penetration of the drying "front", a considerable difference when the temperature passes from -36° to -25° .

(2) The curves do not show any discontinuity which would be indicative of a passage from one step to another in the course of dehydration.

(b) S u c r o s e S o l u t i o n s o f 3 6 % C o n c e n - t r a t i o n (Fig. 7). (1) A comparison of the curves obtained with 36% sucrose at -26° , -36° , -40.5° and -46° with those obtained with 25% sucrose in the same range of temperatures shows the same differences in rate as those mentioned in the preceding paragraph, that is, the rate at -26° is much higher than the rates at -36° to -46° , and the rates within the range -36° to -46° differ little one from the other.

(2) A striking point in the curves shown in Fig. 7 is that the rate at -21° is intermediate between that at -26° and that at -36° , so that there is a reversal in the sequence of rates for a unidirectional sequence in temperatures.

(3) Curves of freeze-drying at -36° , -40.5° and -46° show some straight portions in the intermediate stages of dehydration. The longest and most clearly apparent straight portion is found on the curve for freeze-drying at -46° .

(b) B a n a n a J u i c e (Fig. 8). (1) In contrast to what was observed with sucrose solutions, it is the curve of freeze-drying at the lowest temperature (-36°) which has the highest drying rate, while the three curves at -16° , -26° and -21° differ relatively little one from the other.

(2) However, the rates at freeze-drying temperatures of -16° and -26° decrease much more rapidly with increase in extent of drying than the rate at -36° .

(3) Of the three curves obtained at -16° , -26° and -21° the one which shows the longest straight portion in the intermediate range of "water remaining" is the curve recorded at -21° .

Comments on the Significance of the Data. (a) S u c r o s e S o l u t i o n s o f 2 5 % C o n c e n t r a t i o n (Fig. 6). (1) Let us first note that for a remaining water content in the range between 50 and 40%, the drying rate decreases at the four decreasing temperatures in the ratios 1 : 0.22 : 0.15 : 0.14, while the vapor pressure of ice at those temperatures decreases in the ratios 1 : 0.32 : 0.21 : 0.06. The actual decrease in rate does not, thus, correspond to the decrease in vapor pressure.

Quite evidently the main reason for the great difference in drying rates when 25% sucrose solutions are dried at -26° and when they are dried at lower temperatures is that 26° is above and the other temperatures examined are below the "collapse" point discussed above. But the change brought about by the collapsing of the structure should be of a kind which accelerates dehydration. This effect being just the opposite of that observed on banana juice, we shall discuss it later on, in correlation with the case of banana juice.

Concerning the similarity of the three curves obtained at lower temperatures, we wish to point out that to obtain a constant drying rate with a decreasing driving force (the vapor pressure of ice) one should have a decrease in resistance to the removal of water molecules proportional to the decrease in driving force. One of the factors of the decrease in resistance to the passage of molecules that we actually observed in our studies with the freeze-drying microscope, is the formation of cracks; and these indeed developed more readily at lower temperatures.

(2) The impossibility to distinguish by discontinuities in the curves the passage from primary to secondary drying is probably attributable to the fact that such passage occurs successively at different places throughout the sample and is thus, as a whole, a continuous phenomenon.

(b) S u c r o s e S o l u t i o n s o f 3 6 % C o n c e n -
t r a t i o n (Fig. 7). (1) What has been said of the drying rate
of the 25% solutions freeze-dried at -25° applies here to the case
of freeze-drying at -26° , and what has been said about the reason
for the similarity of the curves obtained at low temperatures also
applies here.

(2) But the lower rate at -21° than at -26° , two temperatures
at which the collapsing should have occurred, is most puzzling. The
discrepancy between the rate and the driving force in those two
curves appears strikingly when one compares the ratios of the rates
to the ratios of the vapor pressures of ice in the 5 curves. They
are, respectively, over the interval from 50 to 40% water remaining:

0.16 : 1 : 0.11 : 0.09 : 0.08

1.64 : 1 : 0.35 : 0.21 : 0.11

It is possible that freeze-drying at -21° results in the release
of a film of solute of greater fluidity than freeze-drying at -26°
and that the sample is somehow sealed off more effectively by this
film.

(3) The straight portion of the curve of freeze-drying at -46°
indicates that the resistance to the vapor flow remains constant when
the depth of the freeze-drying front undergoes a multifold increase.
Such a situation would require a decrease in resistance per unit
depth, which could be accounted for by the formation of cracks
starting in the outer regions and progressing inward with the pene-
trating drying front. The absence of similar straight portions in
the curves obtained at -21° and -26° , where drying is seen in the
microscope to proceed in a very viscous matrix which certainly does
not crack (except in the last stages) is consistent with the argument.

(c) B a n a n a J u i c e (Figs. 8 and 9). (1) The puzzling
reversal in the order of the drying rates when one passes from sucrose
solutions to banana juice in the cases of freeze-drying temperatures
of -26° and -36° , was confirmed in a repetition of the experiments
on banana juice at these two temperatures. The results (Fig. 9)

were practically identical with those obtained with the previous runs (Fig. 8). Additional evidence for the same phenomenon was also obtained with whole banana homogenate which behaved like banana juice.

Quantitatively, the drying rate at -36° is roughly between three and four times as great as the rate at -26° over almost the entire course of drying, while, on the basis of variation in vapor pressure of ice, one would expect the rate at the lower of these two temperatures to be just one third of the rate at the higher.

The reason for the reversal when one passes from sugar solutions to banana juice is almost certainly a difference in the freeze-drying mechanism, as affected by the collapse phenomenon. (The collapse temperature is nearly the same (about -30°) for the juice and for the sugar solutions.) The presence in the juice of proteins, gums and other compounds, in addition to the sugars, is probably responsible for a difference in the collapse behavior, the viscosity of the juice being high compared to that of the sucrose solution. The collapse at -26° possibly causes the matrix to fall against the freeze-drying interface, where it may form a barrier of ever-increasing thickness. By contrast, freeze-drying at -36° would permit the preservation of the system of channels with which the student of freeze-drying is familiar, channels which would allow a more rapid evacuation of the water molecules.

The much greater readiness of the sucrose to freeze-dry at temperatures involving matrix collapse may also be associated with a tendency for the sucrose-rich phase to "puff" more readily than the supernatant-rich phase. Puffing appears to be nucleated by air precipitated from solution during freezing.

(2) The difference in the decrease of drying rates with increase in extent of drying in curves at -36° versus the curves at -26° or -16° is likely due also to the retention of the solute matrix at -36° and to the persistence of a porous structure from which desorption drying can proceed with comparatively little resistance.

(3) The passage from a straight to a curvilinear segment in the curve obtained at -21° probably coincides with the termination of sublimation (or dissolution) of ice. (This tentative conclusion is based on observations made on other cases, which are to be presented in a paper now in preparation.)

(D) Electron Microscope Study of Some Details
of the Freezing Patterns of Sucrose Solutions
which Are of Interest for Freeze-Drying

This study is really concerned with the mechanism of freezing rather than with that of freeze-drying; but, since it was planned as a part of the program of freeze-drying of banana, and since the conclusions we derive from it concern mostly freeze-drying, we include it in Part Two of the report.

Technique. A small quantity of a solution of sucrose in distilled water (10, 20, 30, 35, 40 and 50% sucrose, w/w) was introduced through a fine-tipped pipette into a container of the type described in the previous section (container made of pieces of aluminum foil separated by a split polypropylene ring). The preparation thus obtained was frozen by abrupt immersion in isopentane previously cooled to -35° , -40° , -70° , -100° or -140°C . After the forceps were removed, the preparations were placed on crushed dry ice so that the isopentane could drain, and also to provide a safe means of transport from the room where the samples were frozen to the cold room in which the replicas were to be made.

In the cold room, generally maintained at $-45 \pm 1^{\circ}$, the foil and plastic rings were removed. Each sample was then immersed in a shallow dish of 0.5% Formvar solution (made up at room temperature but filtered just before use, each time, in the cold room at -45°), where a gentle pressure applied along a diameter of the disc-shaped sample sufficed to break it into two or more pieces. The pieces were lifted from the Formvar solution, drained on a piece of clean Whatman #1

filter paper for a few seconds and placed on a second piece of filter paper where they were allowed to dry. After an hour or more, the Formvar coated pieces were trimmed with the aid of a cold razor blade and transferred, on crushed dry ice, as before, to a room at 25° where they were placed, one at a time, on the dust-free surface of a relatively large volume of freshly distilled water. The frozen sucrose solution quickly melted and washed away. Clean Formvar replicas of the surfaces created by cross-fracture, floating independently, were picked up on 200 mesh copper grids precoated with a thin structureless support film (support made of Formvar), transferred to an RCA EMU-3 electron microscope and examined at 50 KV and magnifications from 1,000 to 30,000.

Observations and Their Significance. (a) P h a s e S e p a -
r a t i o n. A point of great importance in the study of the mechanism of freeze-drying is the relative proportion of ice which sublimates freely and of the amorphous solute phase which undergoes the so-called secondary or "pseudo" freeze-drying. The four photographs of Plate 11 illustrate the increase in amorphous phase at increasing concentrations. But as they stand, in different orientations, in unspecified depths within the specimen, and in unknown positions along the temperature gradients, they are really imploring investigators for a systematic quantitative study of their behavior.

(b) P a t t e r n o f C r y s t a l l i z a t i o n. The 40% sample represented in Photo. 4 of Plate 11 shows much smaller ice particles or branches after freezing at -140° than the samples of 30, 20 and 10% concentration (Photos. 3, 2 and 1, respectively). But the suggestion from these photographs that the difference in size is considerably greater from 30 to 40% than from 20 to 30% or from 10 to 20% would need confirmation in strictly comparable conditions.

The two photographs of Plate 13 represent two patterns apparently obtained when the plane of fracture and the basal plane of the crystal

(that is, the plane containing the a axes) were differently oriented with respect to one another; in Photo. 2, the two planes were parallel, in Photo. 1, they cut each other at an angle. If we judge by the frequency of the patterns obtained in our numerous observations, the crystals develop along their a axes more readily than along their c axis, at least in the conditions of rapid freezing of our experiments.

An attempt was made to find the factors responsible for the curvilinear path sometimes followed by growing ice spears or branches. Photo. 1 of Plate 14 shows a few curved branches in a field of predominantly rectilinear ones. No relationship between this feature and the freezing temperature or the concentration could be established, but there was some evidence for a more frequent occurrence of the curvilinear pattern in the center of the preparation.

(c) R e c r y s t a l l i z a t i o n. It has been shown that solutions of sucrose of 30 to 40% concentration, cooled rapidly and rewarmed, become intensely opaque at about -31° (Luyet, 1939) and that the opacity is due to an increase in size of the particles of ice (Luyet and Rapatz, 1958), a phenomenon designated as irruptive recrystallization (Luyet, 1960). It was of interest to examine under the electron microscope replicas of such solutions before and after exposure to the recrystallization temperature. The results are shown in Photos. 1 and 2, respectively, of Plate 12. The increase in size of the ice particles and their separation from one another are conspicuous.

An interesting feature of the recrystallization particles is that several of them have straight edges, indicating that each particle consists of one crystal. (One should notice also straight edges, indicative of flat surfaces, in Photo. 3 of Plate 11, where the preparation was not rewarmed to the recrystallization temperature.)

A particular effect of recrystallization is to cause the segmentation of an ice spear or branch. This phenomenon is illustrated

in Photo. 2 of Plate 14, though in that case, recrystallization was not induced by a rise in temperature.

Photo. 2 of Plate 15 represents another variety of recrystallization pattern. The arrows point to places where the separate branches and subbranches of a developing structural unit meet and fuse. This process is apparently the result of a "spontaneous" recrystallization which occurred in the course of rapid freezing.

(d) P a t h w a y s f o r t h e E v a c u a t i o n o f W a t e r M o l e c u l e s. A fundamental question in the study of the mechanism of freeze-drying is the manner in which the ice is imbedded in the amorphous solute and the extent to which it is surrounded by the amorphous phase. Photo. 3 of Plate 11 shows areas, which apparently represent cross-sections of branches, entirely surrounded by the solute; these branches, which are a part of the network of pathways for the evacuation of the water molecules in the course of sublimation, will generally be capped by the amorphous solute at their growing end, they may or may not be connected with ice stems at the other end. Photo. 2 of Plate 13 shows cases in which connection to the stem is severed. When a recrystallization took place upon rewarming (Photo. 2 of Plate 12), we have evidence with this material and several others, that the recrystallization particles are separate entities.

In several of the photographs, in particular in those of Plate 15, one distinguishes areas or zones characterized by different freezing patterns. Sometimes one can follow the development of one such pattern from an original stem or branch. Thus, most of the structure seen in Photo. 1 of Plate 15 has developed from a stem in the upper right hand corner (at arrow). If, in the course of freeze-drying, all the ice in the territory characterized by one pattern has to be evacuated through its place of origin, such a place would constitute a severe bottleneck.

However, a recrystallization of the type marked by arrows in Photo. 2 of Plate 15 would permit the formation of a system of by-passes to the bottlenecks which would facilitate freeze-drying.

One may have to investigate each particular combination of sample composition, sample geometry, and cooling regime separately in order to determine the likely contribution of such factors to the freeze-drying behavior of the sample.

PART THREE

REHYDRATION OF FREEZE-DRIED PRODUCTS AND MISCELLANEOUS QUESTIONS

I. PROBLEMS AND GENERAL MODES OF APPROACH

The details of the mechanism of rehydration may be as significant for the understanding of the basic phenomena in freeze-drying as the details of the mechanism of freeze-drying itself. It is primarily from that point of view that we studied rehydration.

The miscellaneous questions which called for additional treatments were: (1) the rehydration of freeze-dried products subjected to compression; (2) the effects of freezing after partial dehydration; (3) the effects of freezing on fruits of different states of maturity.

Part Three of this paper is, accordingly, divided into four sections: The first, on fundamental phenomena in rehydration, is based primarily on work with banana, but it includes also some observations made on peach, apricot and spinach. The second on rehydration of compressed freeze-dried products reports the results obtained with corn and peas. The third is concerned with the freezing of partially dehydrated lettuce. The fourth deals with some effects of freezing and freeze-drying on ripe and incompletely ripe banana, tomato and pineapple. We also compared the younger, inner leaves of a head of lettuce with the outer ones.

Of the various problems involved in rehydration, we selected for study the effects of: (1) the biological structure and physical conditions of the product, (2) its chemical composition, (3) the rate of freezing, (4) the temperature of freeze-drying, on the rate and the completeness of rehydration. — The other problems studied were indicated by the words underlined in the list of miscellaneous questions above.

The principal mode of approach used in the study of rehydration consisted in bringing a surface of the specimen in proximity or in contact with water, recording the rate of penetration and observing the effects of obstacles such as nonwettability, entrapped air, etc.

The method of compressing a freeze-dried product involves a slight remoistening before the pressure is applied. We have no experience of our own in that treatment. The material was supplied to us in the compressed form. — For the partial dehydration of lettuce we used either the method of exposure to dry air, or of immersion in hypertonic solutions.

II. RESULTS AND DISCUSSION

(A) Studies on the Mechanism of Rehydration in Banana and Its Components

We used two methods in our studies of the rehydration of freeze-dried materials from bananas. One consisted in applying a droplet of water to a piece of previously freeze-dried material and following the progress of the advancing rehydration "front". In the other method a thin layer of the material was mounted, while still fluid, in grooves between a slide and coverslip; it was then frozen, freeze-dried and rehydrated under conditions of confinement which permitted microscopic observation, photography and cinematography of the progress of rehydration. The observations made on rehydration of other fruits and vegetables will be mentioned in an appendix.

(1) Rehydration of Slices of Material

Frozen and Freeze-Dried in Bulk

Material and Method. Material of four types was rehydrated by this method: (a) banana sliced at room temperature into cylindrical pieces, approximately 1 cm. high, which were frozen in still air at $-30^{\circ}\text{C}.$, freeze-dried at -10° (temperature of specimen chamber); (b) entire banana frozen in still air at -30° , freeze-dried at room temperature; (this material developed many longitudinal and transversal cracks); (c) banana slices frozen and freeze-dried according to the accepted "commercial procedure" (freezing in airstream, freeze-drying on plates maintained at $120^{\circ}\text{F}.$); this material was supplied to us by the Natick Laboratories; (d) Kellogg's freeze-dried banana product (procured from the market).

A droplet of water was applied to the surface of a piece of freeze-dried material, either an untouched surface or one which has been shaved with a razor blade, or one which has been produced by rupture. Then we recorded the wettability of the material and the rate of penetration of water, and took note of the type of resistances encountered,

Observations. (1) Kellogg's reconstituted product wetted instantaneously at all points on original, on shaved, and on fracture surfaces; the rate of penetration was of the order of 1 mm. per second. (2) Slices treated as stated under (a) above, also wetted instantaneously on the three types of surfaces, except for a slight delay at certain points of the shaved surfaces; the rate of penetration of water was also about 1 mm. per second. (3) Samples from the entire bananas (category b) wetted only after water had rested on the surfaces for one or two seconds; certain portions of the surfaces wetted only after some 100 seconds; shaving of the surfaces increased slightly the wettability; the rate of penetration after wetting was of the order of 0.1 mm. per second. (4) Specimens from commercially freeze-dried banana slices wetted in some places instantaneously and in others only after contact with water for several seconds or for longer times; shaving of the surfaces did not bring about any changes in wettability; the rate of penetration varied from 0.1 to 0.01 mm. per second.

Comments. Among the reasons why the cylindrical sections of category (a), freeze-dried at -10° , are more readily wetted than the entire bananas (category b), freeze-dried at room temperature, one may suggest the following: (1) A higher freeze-drying temperature may have caused the migration of small amounts of lipids which would render the material more hydrophobic. This hypothesis is supported by the similarity in wettability in specimens of categories (b) and (c) which were exposed to higher temperatures than specimens of category (a).

The high wettability of the Kellogg's product might be explained by the last-mentioned effect brought about by the processing of the puree before freeze-drying, or by the lowering of the freeze-drying temperature resulting from a higher freeze-drying rate.

A reason for the more rapid penetration of water in sections of category (a) than the entire fruit (category b) may be that the flat surfaces (those perpendicular to the axis of the cylinders) on which freezing may have started in sections of category (a), and on which more numerous channels would be formed upon freeze-drying, permit a readier access to the rehydrating water coming in the direction of the axis (the rehydrating water was placed on the flat surfaces).

From what was just said on the possible effects of the orientation and number of channels on the rate of rehydration, one would expect a high rate in the category (c) in which the bananas were also sliced. The results are just the opposite of that expectation. A factor which is evidently of much greater importance is the "collapse" phenomenon, which is known to occur at about -31° for banana tissue.

The actual temperature at the subliming interfaces was probably between -20° and -30°C . (in the case where the specimen chamber was kept at -10°). When the specimen chamber was maintained at room temperature, the center of the slices probably experienced a brief cooling to -20° and a rise in temperature to -5° or thereabouts before

freeze-drying was completed (actual measurements from thermocouples placed at the center of slices freeze-dried in specimen chambers at room temperature showed this to be the case). Thus there would have been a much more extensive collapse in the samples freeze-dried at room temperature than in those freeze-dried at -10° . More extensive collapse implies migration of the sugary solubles over much greater distances and formation of much more massive barriers to the entrance of the rehydrating fluid.

It is also possible that the structural components of the tissue have their own higher collapse temperature, which was exceeded at the higher freeze-drying temperatures. Such a collapse would involve the migration of particulate cellular components and their aggregation into impermeable barriers.

Still another possibility is that the pectin and protopectin present between the cells exhibit their own collapse behavior — the collapse temperature of every gum we have examined so far falls around -10° — and that the freeze-drying of tissues, presumably injured by freezing, permits, above the T_c for pectin, a displacement of entire cells. Such a collapse, combined with that of the cell constituents could bring about considerable changes in structure.

The ready rehydration of the Kellogg's banana product can be explained by the absence of any cellular elements — neither starch grains nor cellulosic matter was found in this material under microscopic examination — and by the stated use of sodium carboxymethyl-cellulose. The collapse temperature for such a product would have been quite high, probably close to -10° , and the freeze-drying temperature at the subliming interface would be lower than this value because of the extensive evaporative cooling made possible as a result of the low resistance offered by the open structure to the passage of water vapor. (One can only compliment the Kellogg's scientists for devising a product with properties so suitable for freeze-drying.)

(2) Rehydration of Thin Layers of Material Frozen and Freeze-Dried Between Slide and Coverslip

Apparatus, Technique and Material. A new method was devised for the microscopic examination of the mechanism by which the various systems studied underwent rehydration.

The apparatus included a microscope equipped with 3.5 and 10x objective lenses, a motion picture camera equipped with a time-lapse control mechanism, a microsyringe with micrometric feed, and desiccators with silicone O-ring seals.

Two parallel lines (Fig. 10) were drawn on a microscope slide with the tip of a micropipette containing rubber cement diluted with benzene, and two similar lines were drawn on a coverslip. The slide and coverslip were then taped together, after the insertion of copper wires which served as spacers, in the manner shown in the figure.

A small volume of the material to be studied was introduced with the aid of a glass capillary into the space delimited by the four water-repellent lines, which were not wetted by the fluid material. The method is illustrated in the lower part of Fig. 10.

The preparation thus obtained was frozen either in a cold room at -20° or by immersion in isopentane at -140° . It was freeze-dried in a desiccator filled with freshly dried Linde Molecular Sieve (Type 5A) and held in a cold room at either -20° or -40° . As many as forty separate preparations, arranged on edge in plastic microscope-slide storage boxes, were freeze-dried at one time. Freeze-drying was continued for one week at -20° , daily checks being made of the vacuum, and for a month at -40° , with weekly checks on vacuum. (The silicone rubber seals proved to be effective in preventing leaks at the temperatures of the experiments.) The periods during which the samples were freeze-dried were based on previous observations with the freeze-drying microscope; for safety's sake, we tripled the length of time indicated by these observations.

After completion of freeze-drying, the desiccators were transferred to a "dry box" at room temperature, but they were opened to

dry air only after the vessels and their contents had reached room temperature. The preparations were then removed, one at a time, and rehydrated with a droplet of water deposited with the micro-syringe at one end of the channel delimited by the water-repellent lines. (These studies were carried out during January and February, when the relative humidity of the laboratory atmosphere remained in the range from 12 to 20%.)

The material investigated by this method was (a) a dextran solution, (b) banana juice and (c) either the flesh of banana reduced to a pulp, or pieces of banana tissue. The dextran solution (30% w/w) had been frozen by exposure to air at -30°C . and freeze-dried either at -20° or -40° . The banana juice had received one of four pretreatments: rapid freezing (at -150°), rapid freezing and recrystallization, slow freezing (in air, at -30°), slow freezing and recrystallization; in all cases it had been freeze-dried at -20° , a temperature at which "collapse" occurred. The banana pulp and tissue had been rapidly frozen and freeze-dried at -40° , below the collapse temperature.

Observations. (a) D e x t r a n S o l u t i o n. Before re-hydration, the white, porous matrices obtained by freeze-drying appeared to have preserved the elements of structure resulting from freezing; they exhibited very finely dendritic patterns of ice formation (the "coarse spherulites" of Luyet and Rapatz, 1958) in which the original ice crystallites must have possessed diameters of one or a few micra.

When water was applied, it was taken up by the freeze-dried structure with very great difficulty (rate of penetration of the "front": about 1mm. or less per hour), though the exterior surfaces of the samples were instantaneously wetted. Seen under the microscope, the "front", that is the edge of the rehydrated zone, progressed smoothly, though with the same difficulty without regard to the relative directions of the initial freezing and of rehydration; capillary uptake of water was never encountered. The dextran phase became clear and fluid just behind the advancing front and large numbers of very

small bubbles (from a few micra in diameter upward) appeared to emerge from the boundary to the clear fluid medium. These bubbles underwent a continuous reduction in numbers and those remaining grew, ultimately, to as much as 100 μ in diameter. Bubble growth did not, however, proceed by collision and coalescence (this was in fact never observed) but by a process apparently involving the transfer of gases, presumably air, through the solute medium (the air in the smaller bubbles would have been at a higher pressure and hence more soluble).

We tentatively concluded that the rehydration of a very finely structured matrix of a solute of high molecular weight proceeds, not by capillary action, but principally by molecular transfer of water to the dry matrix near the "front", and absorption by the matrix, and that air present in the matrix is not pushed ahead of the front but is entrapped in innumerable tiny spaces and converted to bubbles as surrounding structures hydrate and soften. A more detailed interpretation must await observations at higher magnifications.

(b) B a n a n a J u i c e. Before rehydration, the collapsed material had a sort of alveolar structure, that is, it consisted of a meshwork enclosing empty cavities. Some of the cavities had a diameter of as much as 500 μ ; others were less than a tenth of that size. Some were entirely isolated, others were linked together.

On the basis of the rate of rehydration, one may distinguish three cases, one in which the invasion by water was very rapid, one in which it was rapid but stepwise, and a third in which it was slow.

In the first case (very rapid rehydration) small quantities of water introduced to the exposed edge of the sample were drawn into the interior and absorbed in small fractions of a second. Sometimes one could see, under the microscope, water penetrate the sample by flowing from one vacuole to another. One could also observe occasionally that the walls of the alveoli were dissolved by the invading fluid and disappeared altogether after a few minutes.

In the rapid but stepwise rehydration, the invading fluid moved in rapidly and, when it was halfway across a cavity, it might stop for no apparent reason; then, after a few seconds, it proceeded again rapidly. Apparently some internal surfaces were less easily wetted than others and absorption of water vapor might have been required before the surface in question could be wetted. — In other instances rehydration was rapid to the point where all connected cavities were filled. Further progress then depended on the absorption of water by one or more of the bounding walls. Such processes, which required from several seconds to several minutes, resulted in a softening, a distortion, and finally a dissolution of the walls. The same succession of events was repeated when further intact walls were encountered. Thus rehydration was of an intermittent type.

The third case (very slow rehydration) is characterized by the great resistance to rehydration offered by some parts of the samples in which all of the cavities are isolated by well-formed walls. Rehydration seemed to involve a slow absorption of water by these walls. Irregularly shaped cavities became circular as the partitions between them softened. One should note that the apparently isolated cavities must have contained air, otherwise they would have been eliminated entirely.

A point which calls for particular attention is that, in the cases reported, water travelled molecule by molecule through a solid meshwork and not through the empty spaces enclosed in the meshes. This mode of rehydration is in fact the reverse of the mechanism by which the process is commonly understood to occur.

In general, it appears that, in the case of very rapid rehydration, the only resistance to be overcome was the resistance to the flow of water, and that, in the case of very slow rehydration, two other forms of resistance were encountered, one to the diffusion of the water molecules, the other to the deformation of the structure.

(c) B a n a n a P u l p a n d B a n a n a T i s s u e. Before rehydration, the pulp samples exhibited faint systems of cracks, by which they were divided into platelets (like mud, after exposure to strong sunshine). Within each platelet, starch grains and fragments of cell walls seemed to be uniformly distributed in the otherwise amorphous material. The starch grains scattered so much light that it was difficult to ascertain the presence of dispersed cavities in them. — The tissue samples resembled the pulp samples in their ability to scatter light; they differ from them by the presence, here and there, of some elongated cells.

Rehydration was observed to proceed in four steps: (1) The sample slowly underwent a change in appearance in the presence of water vapor diffusing ahead of the invading liquid. The cracks widened, as if the tensile forces which maintained the shape of the platelets in the dry state were released when the sample sorbed water.

(2) Liquid water brought into contact with one side of the sample wetted the contents of single platelets very rapidly and, in doing so, caused the formation of numerous very small air bubbles. Presumably, the invading water closed the ends of small culs-de-sac or the two ends of channels, thus entrapping the air in them. In pulp in which small pieces of tissue were distinguished, and in the tissue samples, bubbles of more or less rectangular section frequently formed chains, parallel to each other over considerable distances. In no case did we observe any marked resistance to wetting within the platelet itself.

(3) Rehydration was found to be hindered, however, by the interstitial spaces and channels. Water did not appear to wet the outer surfaces of the platelets as readily as their interiors. In fact, considerable periods elapsed between the invasion of one platelet and the wetting of its neighbor and, once water had flowed into an interstitial space, the rehydration of the interior of the adjacent platelets began at once. Water flowed into some interstitial spaces

more readily than it did into others and, in certain cases, whole platelets were thus bypassed and remained unrehydrated.

(4) Changes subsequent to the passage of the rehydrating water front were rather slow. Starch grains were seen to undergo a slow swelling which continued for a short time, occasionally for as much as a minute after passage of the front, and which caused an overall swelling of the platelets to the point where the channels between them were finally eliminated. Presumably the starch grains were merely regaining their original sizes in this process. — A slow redistribution of air took place by migration of small bubbles and their aggregation into large ones.

Appendix

Miscellaneous Observations on the Rehydration Process in Various Fruits and Vegetables

(a) The fact reported above of a softening of the freeze-dried material by water vapor diffusing ahead of the water front could be observed well in the rehydration of thin sections from commercially freeze-dried strawberry and apricot, subsequently mounted between slide and coverslip and examined under the microscope. The more or less angular contours and sharp edges of constituent particles of tissues were seen to round up and swell, while the water front was maintained at a distance.

(b) In the rehydration of pieces of commercially freeze-dried spinach, conditions were particularly favorable for the observation, under the microscope, of the swelling of individual chloroplasts or of aggregates of chloroplasts which, in the dry state, had assumed the shapes of irregular polyhedra. After rehydration, the freeze-dried tissues resembled quite closely the nonfrozen controls; the entire cells, the protoplasts and the nuclei appeared normal.

The main differences between spinach and strawberry or apricot were (1) that the invasion by water was much slower in spinach, (2) that the swelling by water vapor was much more conspicuous in

strawberry and apricot, and (3) that the rehydrated tissues were intact in spinach, while they were disorganized in the two fruits. But, in making such a comparison, one should note that the preparations of spinach examined contained entire cells, whereas the sections of strawberry and apricot contained only thin slices of large cells.

(B) Rehydration of Freeze-Dried and Compressed Products

Materials and Technique. The materials, prepared at the Natick Laboratories, included corn and peas, either merely freeze-dried or freeze-dried and compressed into compact rations.

Our task was to make preliminary tests on their behavior upon rehydration. Our methods consisted in observing the rate of absorption of a droplet of water placed on a surface cut across the seed, and in determining the capacity of the material for imbibing water.

Observations. (a) C y t o l o g i c a l F e a t u r e s. Under the microscope the endosperm of freeze-dried (not compressed) corn is seen to consist of large cells filled with starch and separated by large intercellular spaces. In the center of the kernels are large parenchyma cells. — In compressed corn, the cells have been crushed and are hard to distinguish. One may, however, observe that they have been reduced to about one-third of their original dimension in the direction of the compression. The intercellular spaces are almost completely absent.

Upon rehydration, the cells of the endosperm of corn which has been only freeze-dried increase in volume and thus partly invade the intercellular spaces. The areas previously occupied by parenchyma cells are often empty. There is only a slight overall enlargement of the volume of the kernels. — In compressed corn, hardly any cell can be recognized after rehydration. The overall increase in volume is considerable.

(b) W a t e r A b s o r p t i o n C a p a c i t y. Quantitative determinations of the water content of corn after rehydration was found to be 54.2% (wt./wt.) in the noncompressed sample and 37.3% in the compressed one. A second run gave 53.7% and 36.4%, respectively. (After rehydration the samples had been rolled gently on blotting paper to permit the removal of the excess water; then, they were weighed.) Thus, it appears that compression yields a product which absorbs less water than a sample merely freeze-dried.

A preliminary test, with peas, of the amount of water retained when the rehydrated material was squeezed by the application of a given pressure indicated that the compressed specimen retained slightly more water than the specimen only freeze-dried. If confirmed, these observations may be of significance for the study of the mode of attachment of water to the substrate under the conditions of the experiments.

Another observation of possible significance is that a droplet of water placed on the cut surface of a kernel of corn which had been only freeze-dried was absorbed immediately, whereas the absorption was considerably slower on the surface of a compressed block.

(C) Effects of Freezing on Partially Dehydrated Lettuce

The object of the present study was to determine whether, and to what extent, a preliminary partial dehydration or the introduction of some cryoprotective agent would prevent or lessen the harmful effects of freezing.

Techniques. Dehydration was accomplished either by exposure to the air at room temperature or osmotically in hypertonic solution.

For the air-drying experiments we initially used larger pieces of leaf about an inch on a side, and measured their water loss by weighing them on an analytical balance. Pieces of this size tended to dry nonuniformly, however, probably because of the nonuniform

distribution of vascular elements, some areas becoming dry and brittle while others are still pliable and seemingly moist. Accordingly, we turned to the use of much smaller pieces, some 3 mm. on a side, which we dried suspended from a thin-wire hook on a Roller-Smith balance of 5 mg. capacity.

For osmotic dehydration we used solutions of sucrose and glucose, and for cryoprotection, solutions of glycerol, ethylene glycol and polyvinyl pyrrolidone (PVP).

The partially dehydrated or cryoprotected specimens were frozen either in liquid nitrogen for 1 minute or in a bath at -20° for 10 minutes and were rewarmed rapidly in a liquid bath at room temperature: tap water in the case of air-dried specimens, and the respective hypertonic or cryoprotective solution in the case of osmotically dehydrated or of cryoprotected ones.

Observations. (a) S t r u c t u r e o f L e t t u c e L e a f .

The lettuce leaf, like other leaves, consists of an upper and a lower epidermis, both highly cutinized, with some 5 to 8 rows of mesophyll cells sandwiched between them. The flat epidermal cells vary widely in shape and, except for the guard cells, are devoid of chloroplasts. The nuclei do not appear in the living, uninjured tissue.

The mesophyll consists of so-called "spongy tissue" throughout, that is, loosely arranged cells of various shapes and sizes, with numerous air spaces between them. Most of the space in the mature cells is occupied by the central fluid-filled cell-sap vacuole. This is surrounded by a thin layer of cytoplasm containing the chloroplasts. In section or in an optical plane, the chloroplasts are arranged single-file around the periphery, just inside the cell wall.

(b) E f f e c t o f F r e e z i n g o n F u l l y H y d r a t e d L e t t u c e . Fully hydrated head-lettuce leaves contain some 95% water — values ranging from 94.6 to 95.7% were obtained in a series of determinations. (Higher values were found in another determination, as reported previously.)

The effect of freezing on fully hydrated lettuce has already been discussed (Part One). Suffice it to say here that pieces of fully hydrated lettuce leaves freeze intracellularly in distinct flashes at relatively high subzero temperatures. When such frozen leaves are thawed either rapidly or slowly they become limp, flaccid, and discolored, as if they had been scalded, and do not subsequently regain their turgor.

(c) Effects of Freezing on Air-Dried Specimens. Specimens air-dried till they had lost up to 80-90% of their original weight, but not frozen, recovered completely when they were rehydrated in tap water. Both epidermis and mesophyll seemed normal. Severe as this dehydration appears, it must be remembered that, for material with 95% water content, a 90% weight loss reduces the overall water content to only 50%. Hence the complete recovery is not too surprising.

Pieces dried to this extent froze spontaneously at -20° and of course, also in liquid nitrogen. When thawed they were flaccid and scalded-looking. Their cells appeared collapsed, especially the mesophyll, and internally disorganized, with clumped, seemingly shrunken cytoplasm and chloroplasts, and often well-visible nuclei.

Specimens which had lost more than 90% of their original weight were finely crinkled, due apparently to the collapse of their mesophyll. They generally remained crinkled and flaccid, whether frozen or not.

(d) Effects of Freezing on Osmotically-Dehydrated Specimens. Treatment with sugar solutions, sucrose or glucose, produced marked plasmolysis in both epidermal and mesophyll cells. When a leaf plasmolyzed in this way was carefully blotted and then frozen, the cell contents remained contracted, but generally developed a granular and less smooth-contoured appearance. Frequently the protoplast seemed to have been ruptured in at least one area. The picture, after freezing and thawing, was more that of clumping and coagulation than of plasmolysis.

Severe internal breakdown was also suggested by the fact that active Brownian movement set in almost immediately after thawing. The chloroplasts, especially in the guard cells, looked granular and exploded and the nucleus was often well visible.

No gross damage was discernible in the cell walls nor in and around the vascular bundles. We never succeeded in deplasmolyzing the frozen-and-thawed cells. Nonfrozen cells deplasmolyzed well and assumed a normal appearance.

(e) Effects of Freezing on Cryoprotected Specimens. Cells treated for 15 minutes to 1 hour in solutions of 10% PVP, or ethylene glycol or glycerol appeared turgid and practically normal. Freezing of such specimens resulted in a disruption of the structure, especially of the chloroplasts which often exploded. Cells treated with ethylene glycol showed less injury immediately after freezing than did those treated with PVP or glycerol. The guard cells of frozen-thawed specimens could be plasmolyzed with a 1M sucrose solution but not subsequently deplasmolyzed.

The mesophyll cells which looked practically normal immediately after thawing underwent a gradual irreversible disorganization during the next few hours.

It would appear, therefore, that partial dehydration is not the answer to the problem of the prevention of freezing damage in lettuce leaves.

(D) Effects of Freezing and Freeze-Drying on Fruits at Different States of Maturity

Tests on possible differences in the mode of freezing and of freeze-drying, and in the resistance to injury by freezing and by freeze-drying, were made on ripe and unripe tomato, pineapple and banana. In the three cases, the stage designated as unripe was that

in which the fruit still had green areas which would generally have caused rejection by housewives.

The mode of freezing, in particular the sudden formation of intracellular ice, the behavior after thawing, the response to the permeability test, was about alike in the ripe and unripe tomato. The turgor may have been more pronounced in the unripe one, which had a firmer consistency, but that difference could not be correlated with any effect of the treatment applied. The turgor tests reported in Plate 4 were made on unripe tomatoes. — The behavior of ripe and unripe pineapple was also about the same.

The higher sugar content in ripe bananas was expected to exert an influence on the mode of freezing, on the mode and rate of freeze-drying and on the mode and rate of rehydration. In fact, however, no difference could be ascertained in any of these processes.

Particular attention was given to the collapse temperature, which we also found to be the same no matter how advanced is the maturation process.

In the course of microscopic observations during freeze-drying and during rehydration, we noted the greater abundance of starch in the unripe banana, but this fact could not be associated with any process of cryobiological significance.

Apparently, the two stages of maturity selected in these experiments were not sufficiently different to permit the observation of noticeable differences in either the freezing and freeze-drying processes or in their effects.

In the case of lettuce there are marked cytological differences between the near-meristematic tissues in the central regions of a head and in the tissues of the outer leaves. In the central regions the cells are smaller, they have larger nuclei which contain one or two prominent nucleoli, their cytoplasm is finely granular with occasional vacuoles, they contain only a few scattered chloroplasts.

But after freezing and thawing, and after freeze-drying the damage done appeared to be about the same in the fully developed and undeveloped tissue.

GENERAL CONCLUSIONS

I. MECHANISM OF FREEZING.

(1) In slowly cooled samples at high sub-zero temperatures, three steps could generally be distinguished in the invasion of the tissues by ice: a superficial spreading, the occupation of the intercellular spaces and the penetration into the cells. The latter occurred in sudden "flashes". In rapidly cooled samples, fine-grain ice was quite uniformly distributed throughout the tissues.

(2) Between -10° and the melting point, a recrystallization took place: some of the ice particles grew larger at the expense of smaller ones.

(3) The amounts of ice formed within a few degrees below the freezing point (in the range of -2° to -5°) represent generally some three-fourths or more of the water freezable at any temperature. The amounts formed at -20° seem to approach an asymptotic value. The curves representing the percentage of water remaining unfrozen at various temperatures contain significant information on the water-binding properties of the solid components present; but this field is still unexplored.

II. EFFECTS OF FREEZING AND FREEZE-DRYING.

(1) Slow freezing (at the rates generally used in commercial procedures) results in a considerable disturbance of the cellular structures. Relatively large blocks of ice are formed within the tissues; the cellular framework is often ruptured. Rapid freezing (by immersion of small pieces in liquid nitrogen) does not appreciably disturb the cellular structure. Apparently the ice crystals are too small to produce major disturbances.

(2) Both slow and rapid freezing result in the death of the tissues, as indicated by permeability and turgor tests. (One may remark that little is known on the relationship between preservation of vitality and preservation of food qualities.)

(3) The state of preservation of the structures in freeze-dried material depends primarily on the mode of freezing, except for the phenomenon of "collapse" which will be discussed in the following sections.

General Conclusion: Since rapid freezing and low temperature freeze-drying result in a better preservation of structures (though not of vitality), the answer to the question of desirability of using higher freezing rates for food preservation depends on the answer to the question of the relationship between preservation of structure and preservation of food qualities.

III. MECHANISMS OF FREEZE-DRYING.

(1) The freeze-drying rates for sugar-rich systems at different but constant ice-interface temperatures are not, in general, in proportion to the vapor pressures of ice at the temperatures in question. Lower temperatures may actually yield higher freeze-drying rates. Furthermore the shape of the curve representing the dependence of decreasing weight on increasing time (at constant temperature) varies markedly with the freeze-drying temperature.

(2) Freeze-drying of banana juice (supernatant fluid) at temperatures higher than -30°C . and of pulped banana tissue at temperatures higher than -25°C . occurs with simultaneous "collapse" of the solute matrix, that is, of the three-dimensional framework composed of solutes and solids which, in the frozen state, separates the ice crystals. Freeze-drying at lower temperatures (than -30° and -25° , respectively) proceeds with "retention" of the matrix structure. That is, the geometric distribution of solutes resulting from freezing is preserved. Thus, there is, for each material, a change in freeze-drying mechanism with change in freeze-drying temperature.

(3) The change in mechanism occurs quite suddenly over a change in freeze-drying temperature of only two or three degrees and the temperatures in question seem to be characteristic of the materials not of the previous freezing treatment, nor of the conditions of storage in the frozen state before freeze-drying. The temperature-dependence of the mechanism resembles that observed in many other systems studied in our laboratory and composed of solutions of noncrystallizing substances.

(4) "Collapse" during freeze-drying may seal off potential routes for the escape of water vapor, thus reducing the freeze-drying rates.

(5) Freeze-drying at very high temperatures may cause the temperature of the ice interface to rise so near to the melting point of the system that the collapse temperature of organized elements of tissues is exceeded, causing gross collapse of cellular frameworks (i.e., gross shrinkage or distortion of the whole sample).

It is concluded, on the basis of a comparison of the results obtained in the present study of freeze-drying of banana tissue and of the supernatant tissue homogenate with those obtained in previous studies, in our laboratory, of the freeze-drying behavior of many other substances, that the peculiar resistance of banana and its derivative fluid to freeze-drying lies in the particular combination of sugars and gums present in such large quantities in that fruit.

IV. MECHANISM OF REHYDRATION.

(1) Gross observations on the rehydration of 1 cm.-thick slices of banana, freeze-dried in various ways, showed that the ease with which the surfaces were wetted and the ease with which water penetrated the tissues thereafter depended on the freeze-drying treatment. Thus (a) the wettability was increased when the freeze-drying specimen-chamber temperature was lower (samples freeze-dried at -10° were wetted instantly); (b) the rate of penetration of

water into wetted samples was increased when the freeze-drying temperature was lower, presumably because of a lesser "collapse" under these conditions.

(2) Observations on the rehydration of thin samples in a specially designed microscope assembly demonstrated the dependence of the rehydration behavior on the structural features of the freeze-dried system. Thus (a) a very finely structured matrix resulting from the freeze-drying of thin layers of rapidly frozen dextran solutions at a low temperature proved to be very highly resistant to rehydration; (b) structures resulting from the freeze-drying of sugar-rich solutions, with attendant collapse of the matrix, were difficult to rehydrate, except where interior spaces happened, by chance, to be connected directly with the outer surface of the sample.

One may tentatively conclude (the sample sizes and the methods used prevented us from making direct confirmatory tests) that samples frozen slowly so as to yield large ice crystals, freeze-dried at temperatures permitting "retention" of the original matrix, would rehydrate more rapidly than samples treated in any other way. Such a conclusion would only hold, however, when crystallization started at the sample surface (not beneath it), so that the channels formed by the sublimation of ice are actually connected with the exterior of the sample. If it is so, it would seem important, prior to freezing, to prevent the exterior of the sample from drying to the point where a "skin" formed and precluded the possibility of nucleation at the very surface.

In that connection, one may suggest that a more rapid rehydration would be achieved if some way were found to make sure that channels formed by the sublimation of ice are open at both ends when freeze-drying is completed. It appears as important to leave a way for the air to escape as it is to provide a route for the water to enter.

V. MISCELLANEOUS QUESTIONS.

(1) The question raised above on the relationship between preservation of structure and preservation of food qualities strikes the observer who witnesses the great disturbance caused by compression though the food qualities appear to be satisfactorily preserved.

(2) A dehydration of lettuce leaves until they lost 80 to 90% of their weight does not seem to affect them; they appear normal after rehydration. But freezing the dehydrated leaves damages them. Thus, partial dehydration does not seem to be the answer to the problem of preventing freezing injury.

(3) Preliminary tests on the effects of freezing and freeze-drying fruits at different states of maturity did not reveal any appreciable difference in the limited range of ripeness tested.

APPENDIX

Suggestions for Further Research

- (1) Prevent partial drying of samples prior to freezing (this will yield frozen samples of highest possible water content, with largest ice crystals having most numerous interconnections).
- (2) Freeze slowly in order to create large ice crystals, then lower the temperature and maintain it low in order to prevent recrystallization from breaking the ice phase into many disconnected particles.
- (3) Freeze-dry at low temperatures to avoid (or to minimize) "collapse" (this will preserve the continuous channels created by sublimation of ice and permit a free passage of water vapor to the sample surface).
- (4) Store freeze-dried samples at the lowest possible temperatures (this will prevent the surfaces of the dry product from acquiring water-repellant properties).
- (5) Rehydrate under vacuum, or water-soluble gas such as CO_2 (this will minimize problems arising from the absence of escape routes for air upon entry of rehydrating fluids).
- (6) Search for "collapse" behavior and for characteristic "collapse temperatures" in the cell wall systems of plant tissues freeze-dried after various freezing treatments.
- (7) Examine the plant cell membranes with the electron microscope for holes which might permit direct transmission of water vapor from cell to cell during freeze-drying (holes might arise in the membranes as a result of freezing injury).
- (8) Examine the distribution of ice and the damage to the structure of the tissue in pieces of fruit by the freeze-fracture carbon replication technique followed by electron microscopy.
- (9) Consider the use of a method for "Limited Freeze-Drying" recently devised in this laboratory by Dr. A. P. MacKenzie (paper in preparation).

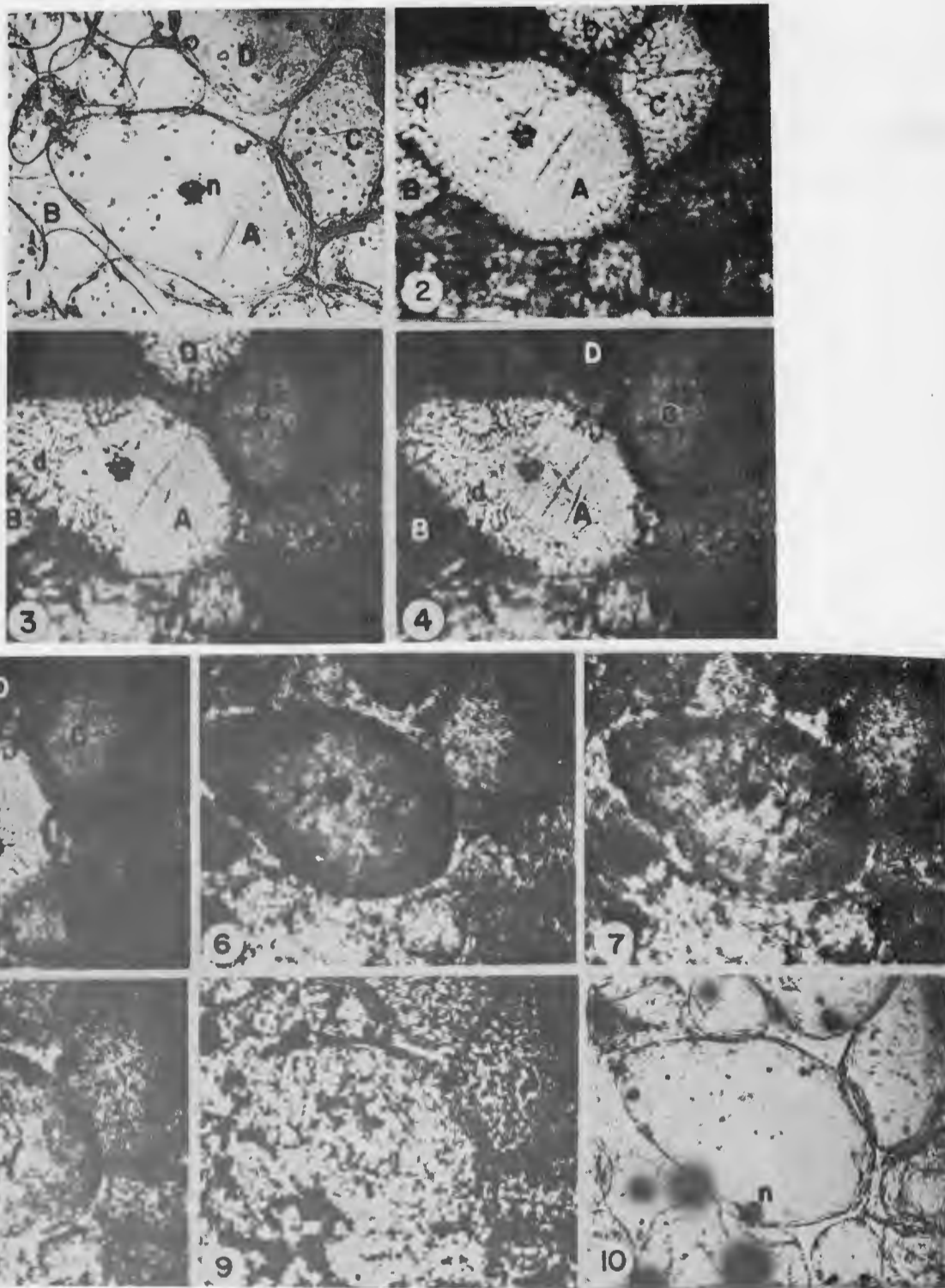


Plate 1. Photomicrographs, taken at 5- to 10-second intervals, showing successive stages in the freezing (superficial, inter- and intracellular) of parenchymatous tissue of tomato. — See text for meaning of symbols. — Magnif.: 80X.

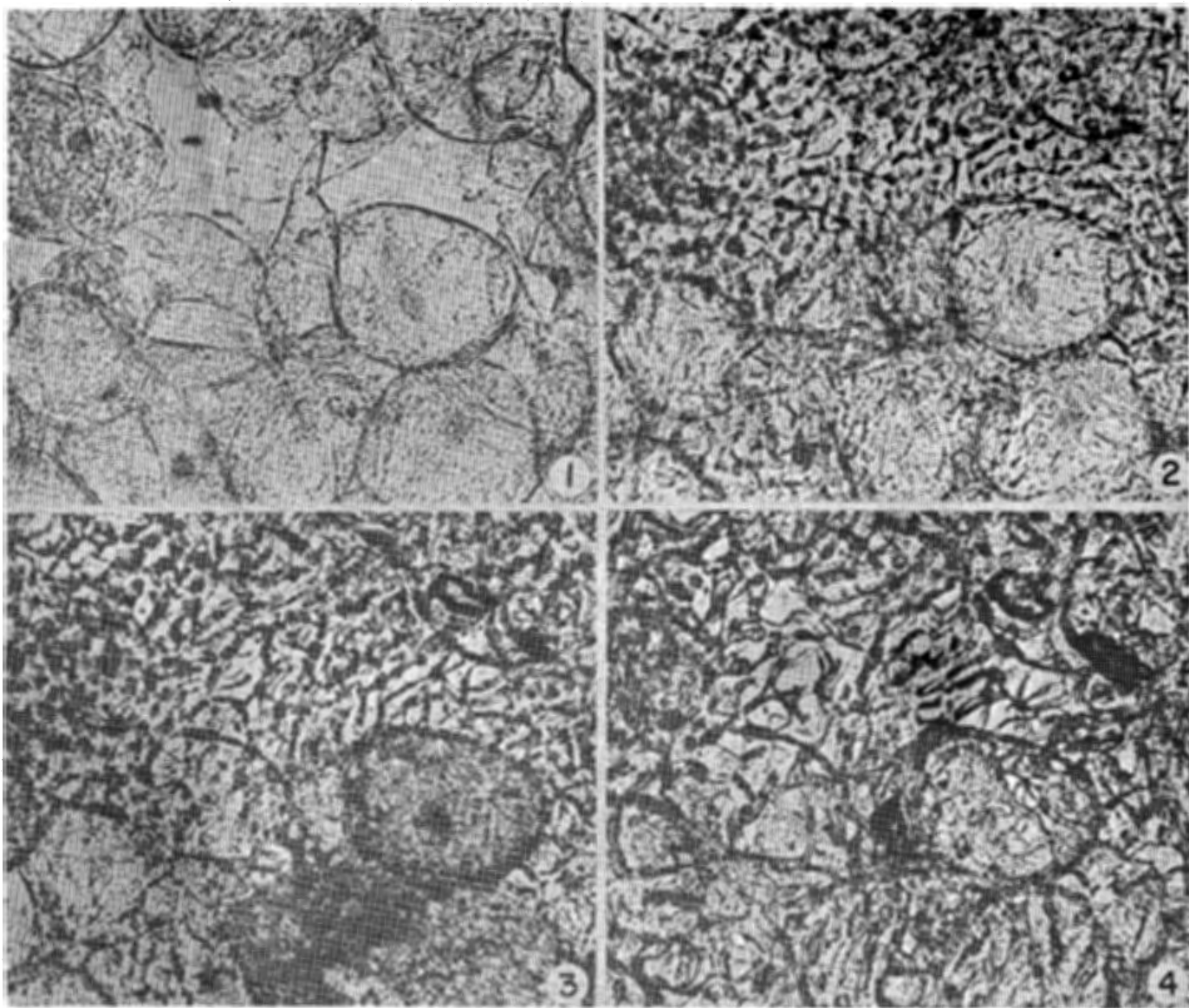


Plate 2. Photomicrographs showing freezing and recrystallization in thin slices of tomato frozen and maintained at -5°C . Photo. 1: Nonfrozen specimen; Photo. 2: Superficial extracellular freezing; Photo. 3: Intracellular freezing; Photo. 4: Recrystallization of intra and extracellular ice after 10 minutes at -5° . Magnif.: 100X.

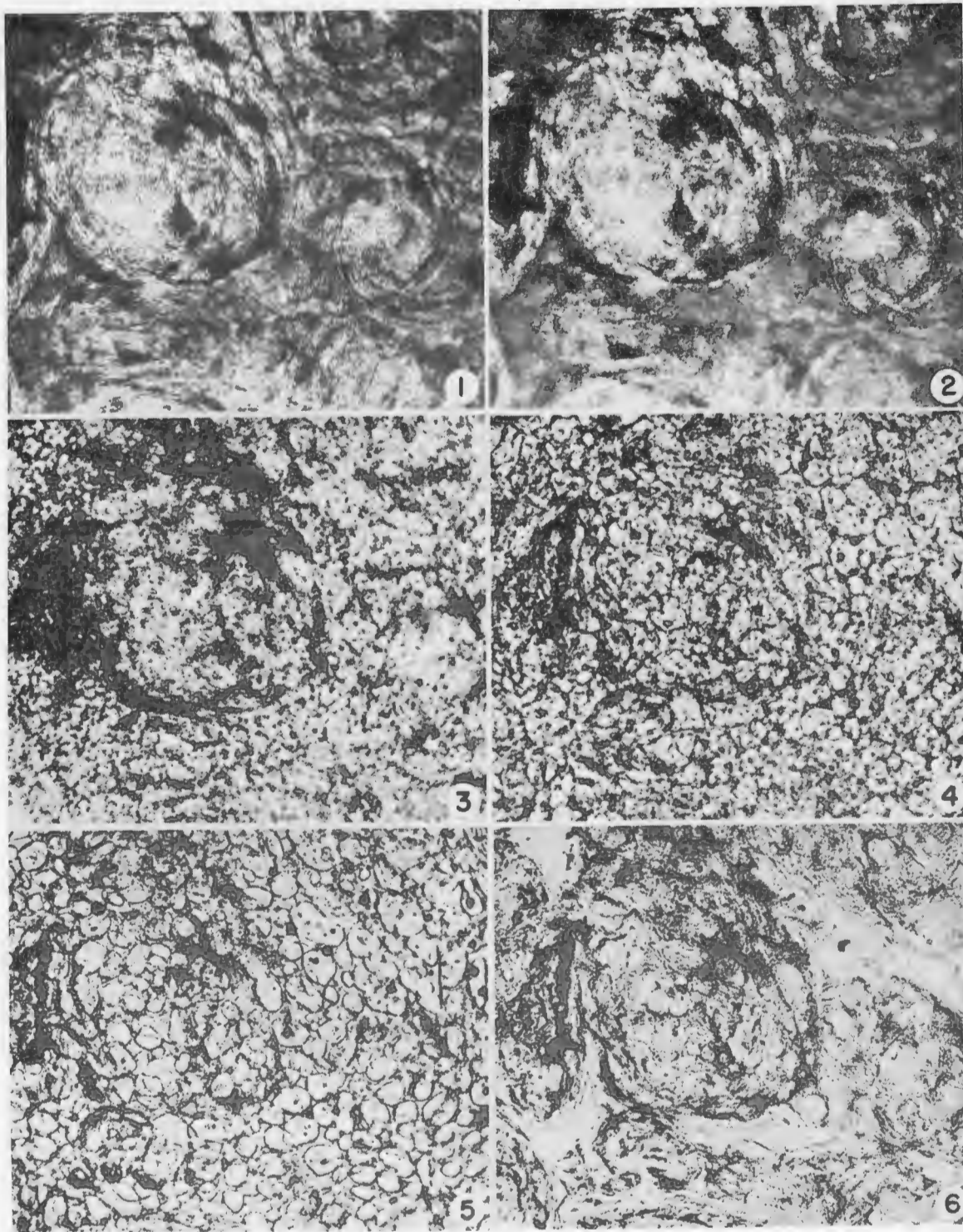


Plate 3. Photomicrographs showing recrystallization in thin slices of tomato rapidly frozen and rewarmed to various temperatures. Photo. 1: Specimen frozen rapidly at -120°C . Photos. 2 to 6: Same field as in Photo. 1 after the temperature was raised to -30° (Photo. 2), to -10° (Photo. 3), to -5° (Photo. 4), to -3° (Photo. 5), where it was maintained for 5 minutes (Photo. 6). Magnif.: 100X.

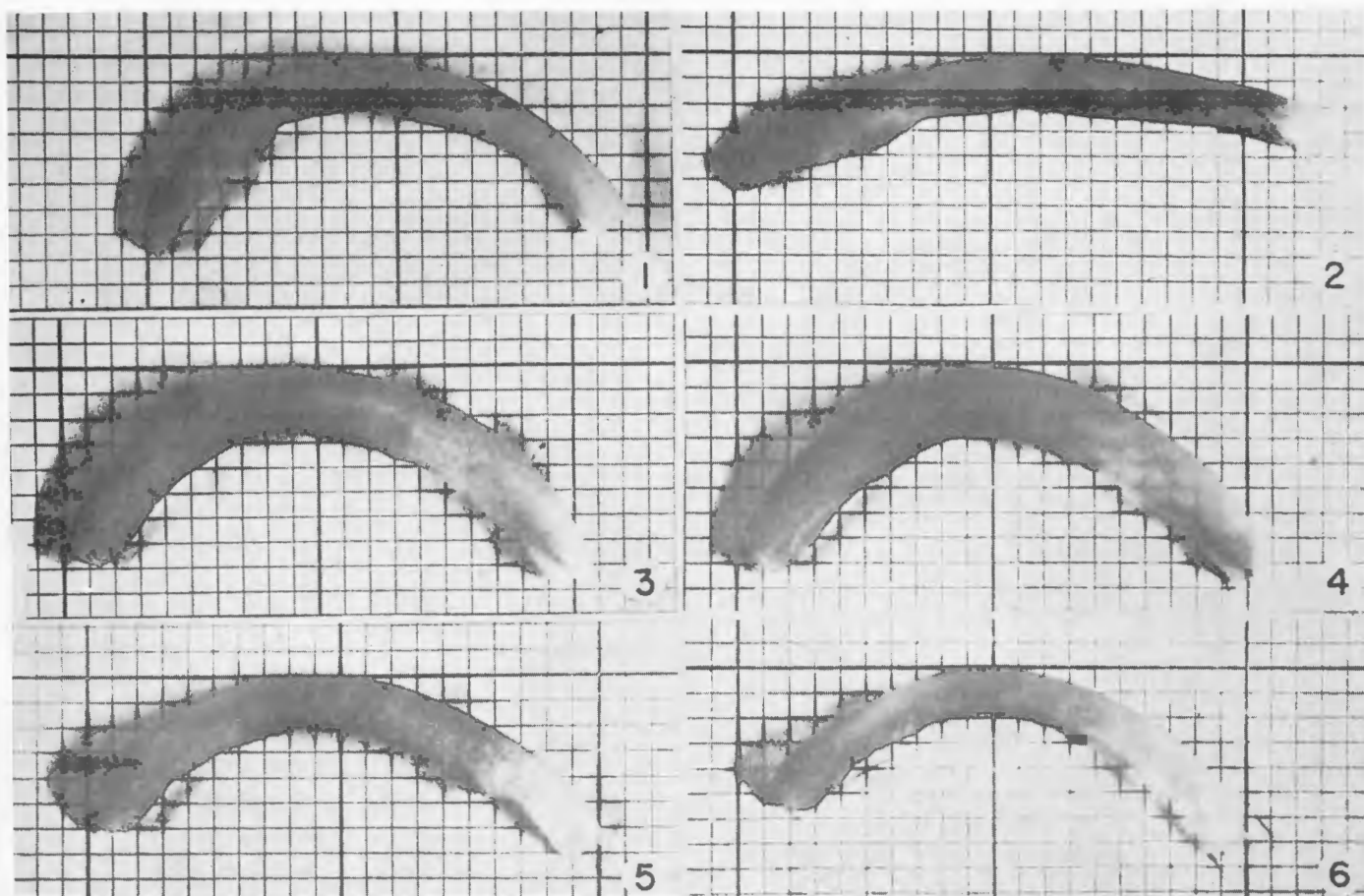


Plate 4. Wedge-shaped pieces of tomato subjected to the turgor test by immersion in distilled water. — Photos. 1, 3 and 5: Controls, not frozen. Photos. 2, 4 and 6: Pieces shown, respectively, in Photos. 1, 3 and 5, after immersion in distilled water, without any previous treatment (Photo. 2), after freezing at -5° and thawing (Photo. 4), and after freezing in liquid nitrogen and thawing (Photo. 6). — Magnif.: 1.3X.

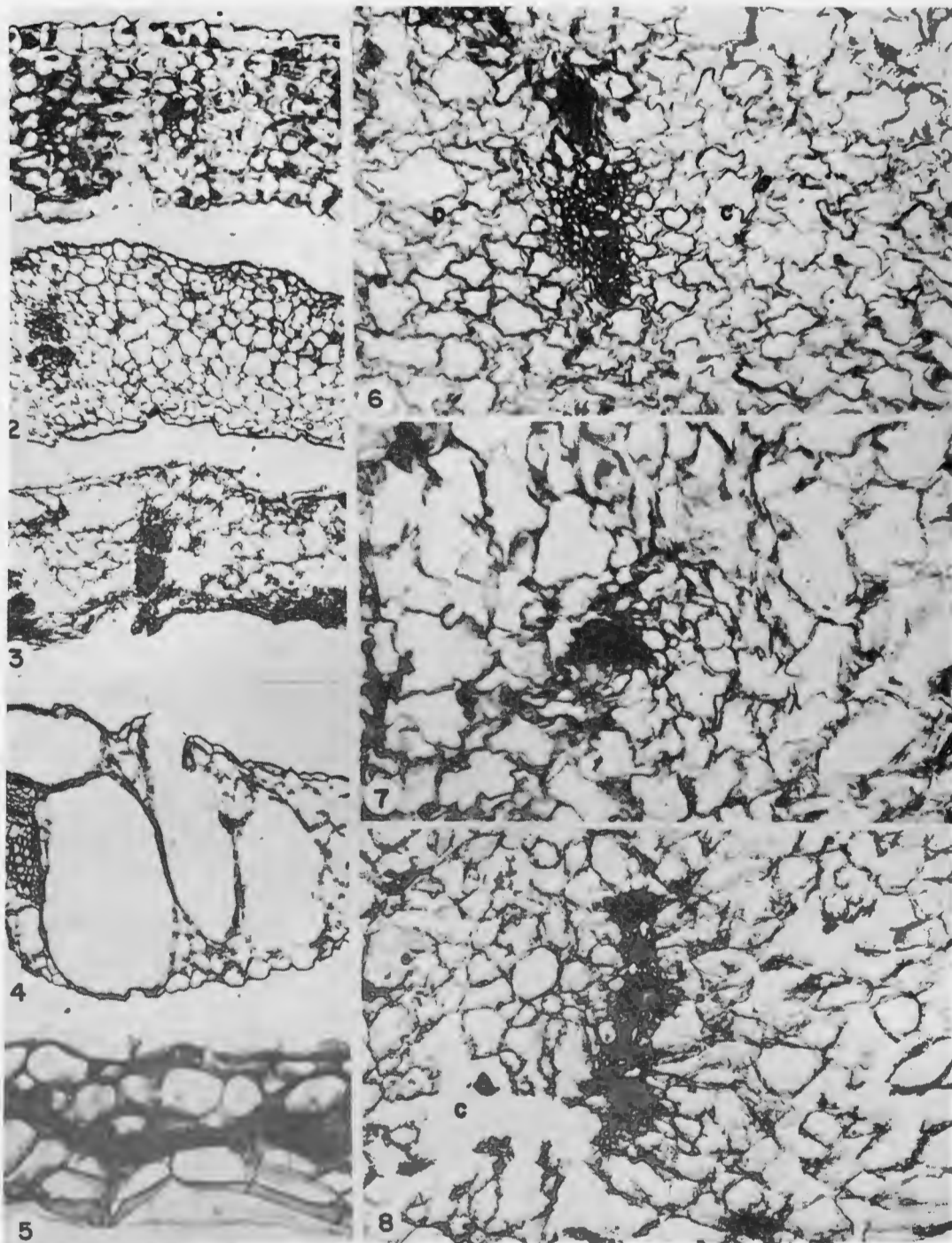


Plate 5. Photomicrographs of freeze-dried lettuce and strawberry tissues. — Photos. 1-5: Lettuce: 1: Nonfrozen control; 2: Specimen frozen rapidly at -150° ; 3: Specimen frozen slowly at -20° ; 4 and 5: Specimens of a commercially freeze-dried head of lettuce. — Photos. 6-8: Strawberry: 6: Nonfrozen control; 7: Specimen frozen rapidly at -150° ; 8: Specimen frozen slowly at -20° . — See text for meaning of symbols. — Magnif.: Photos. 1-5: 90X; Photos. 6-8: 95X.

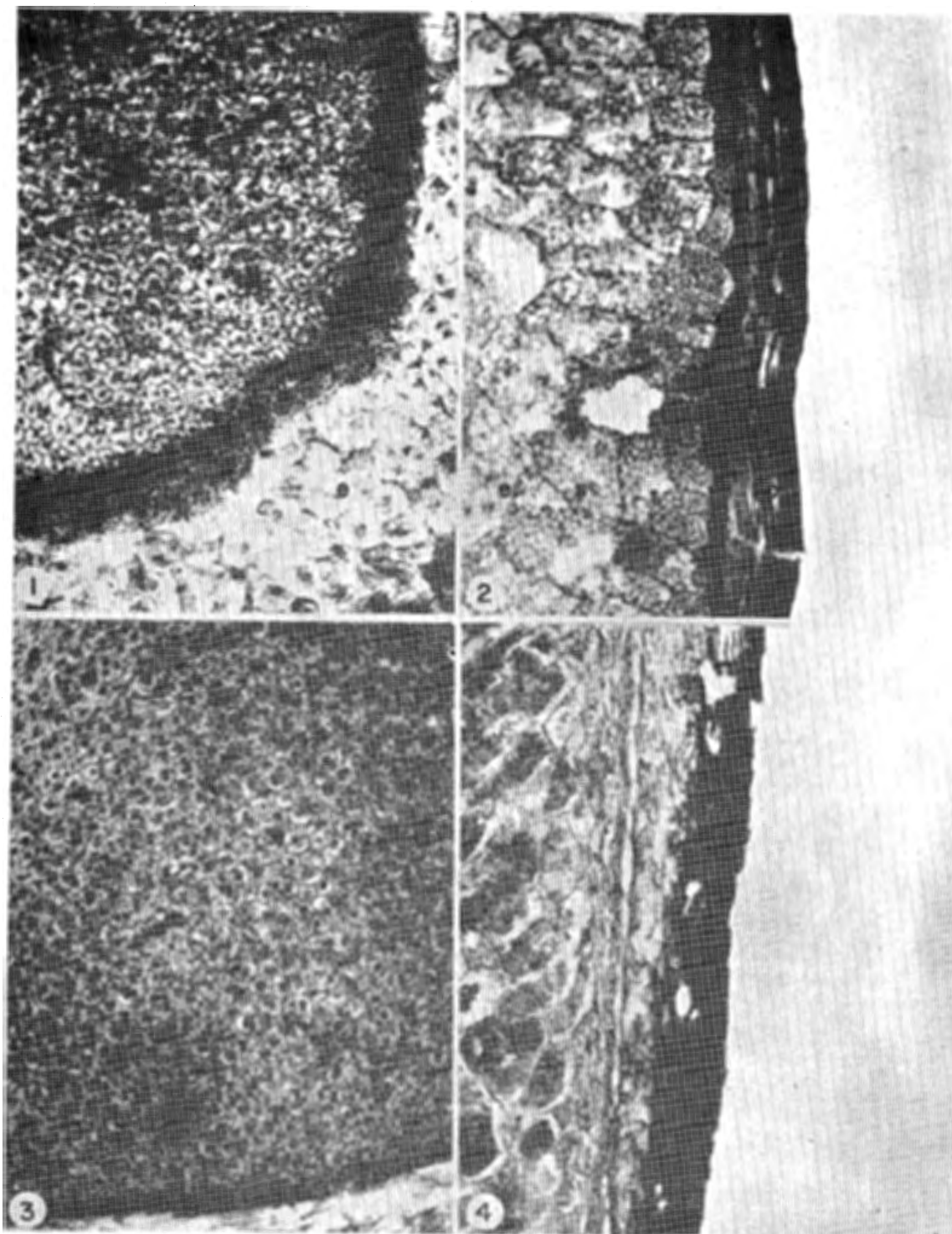


Plate 6. Photomicrographs of corresponding regions of nonfrozen and of rapidly frozen, freeze-dried corn kernels. — Photos. 1 and 2: Nonfrozen control; Photos. 3 and 4: Specimen frozen rapidly at -150°C and freeze-dried at -40° . — See text for meaning of symbols. — Magnif.: 100X.

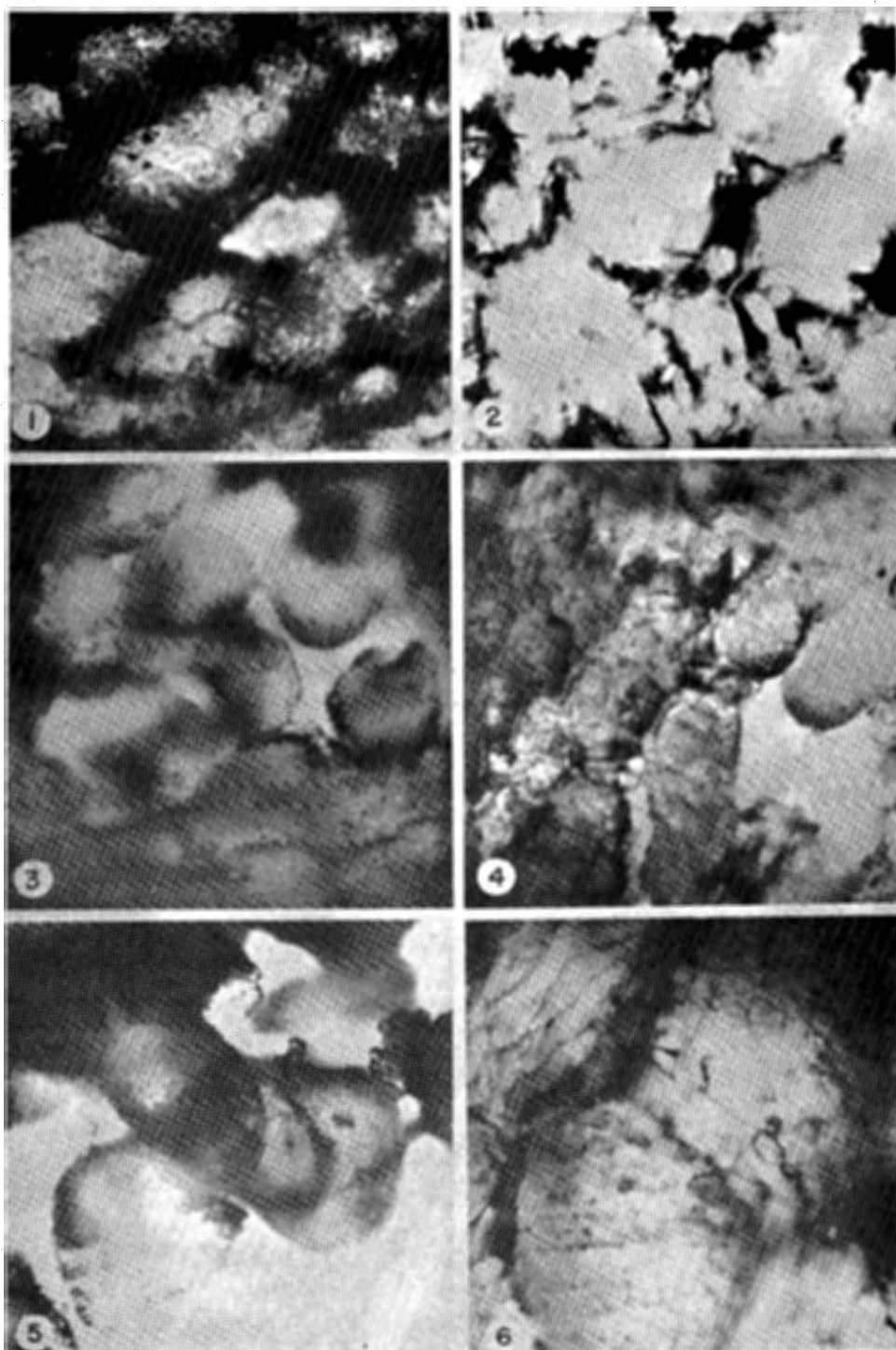


Plate 7. Photomicrographs of parenchymatous tissues of peach, strawberry and banana in the frozen state. — Photographs on the left are of tissues frozen rapidly at -150°C ; on the right, of tissues frozen slowly at -20° . — Photos. 1 and 2: Peach; Photos. 3 and 4: Banana; Photos. 5 and 6: Strawberry. — Magnif.: 110X.

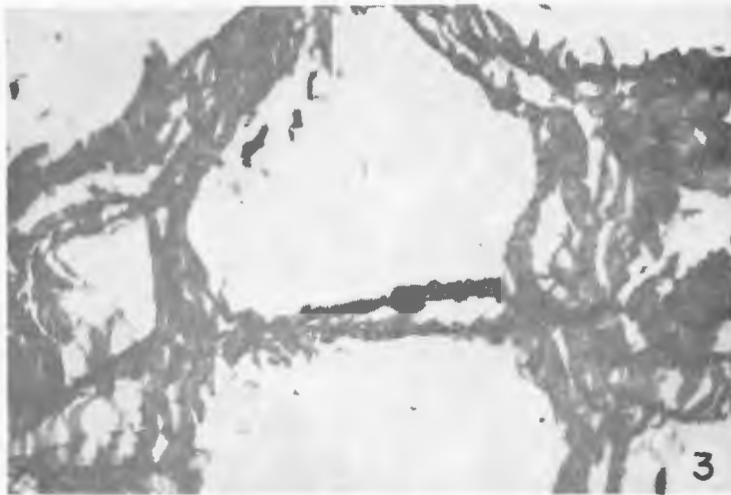
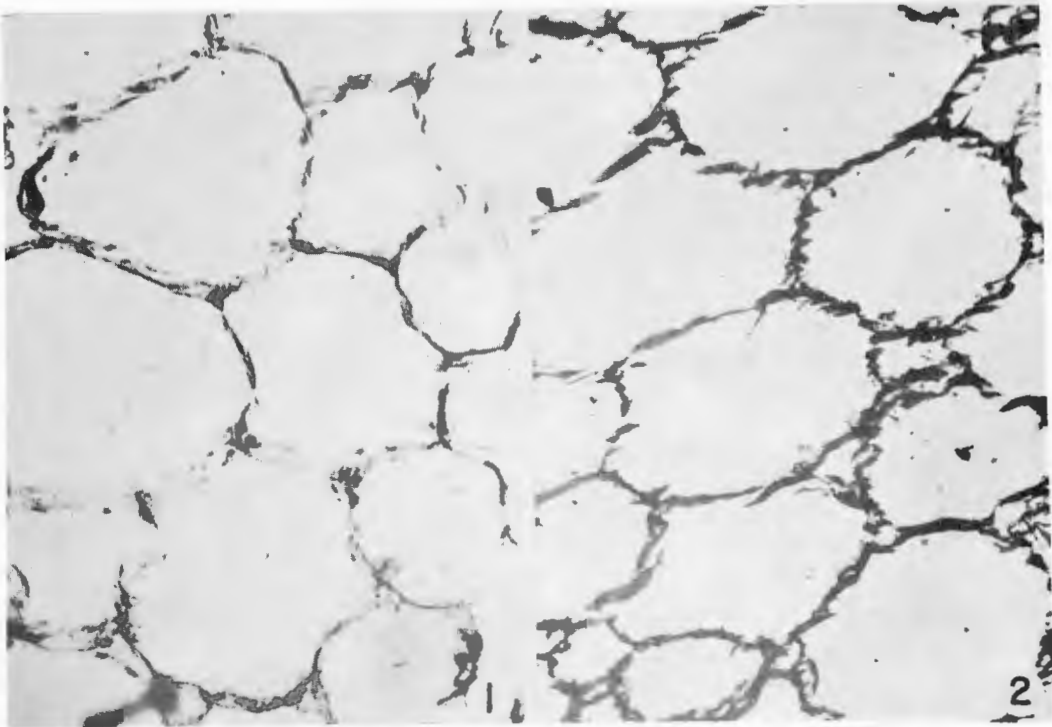


Plate 8. Photomicrographs of thin sections of paraffin-embedded tomato which had been frozen at various rates and then freeze-dried at -40°C . — Photo. 1: Nonfrozen control; Photo. 2: Specimen rapidly frozen in an isopentane bath at -150° ; Photo. 3: Specimen slowly frozen in air at -20° . Magnif.: 100X.

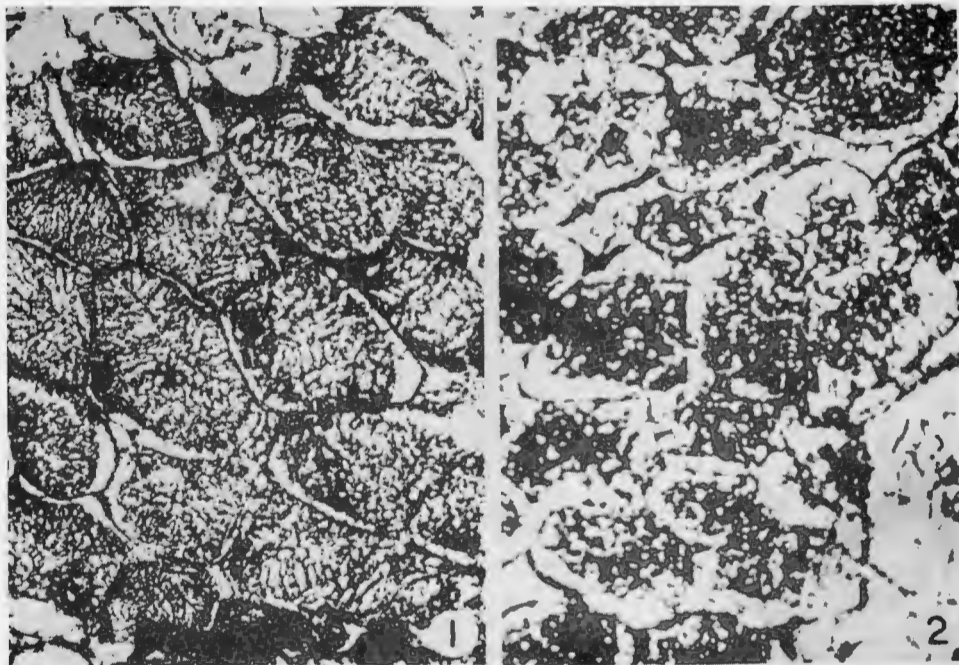


Plate 9. Photomicrographs of thin sections of corn endosperm illustrating the large annular ice cavities between the cell wall and the cell contents and the small ice cavities within the cytoplasm, when the material was examined after having been freeze-dried (Photo. 1), or while in the frozen state (Photo. 2). — Magnif.: Photo. 1: 140X; Photo. 2: 210X.

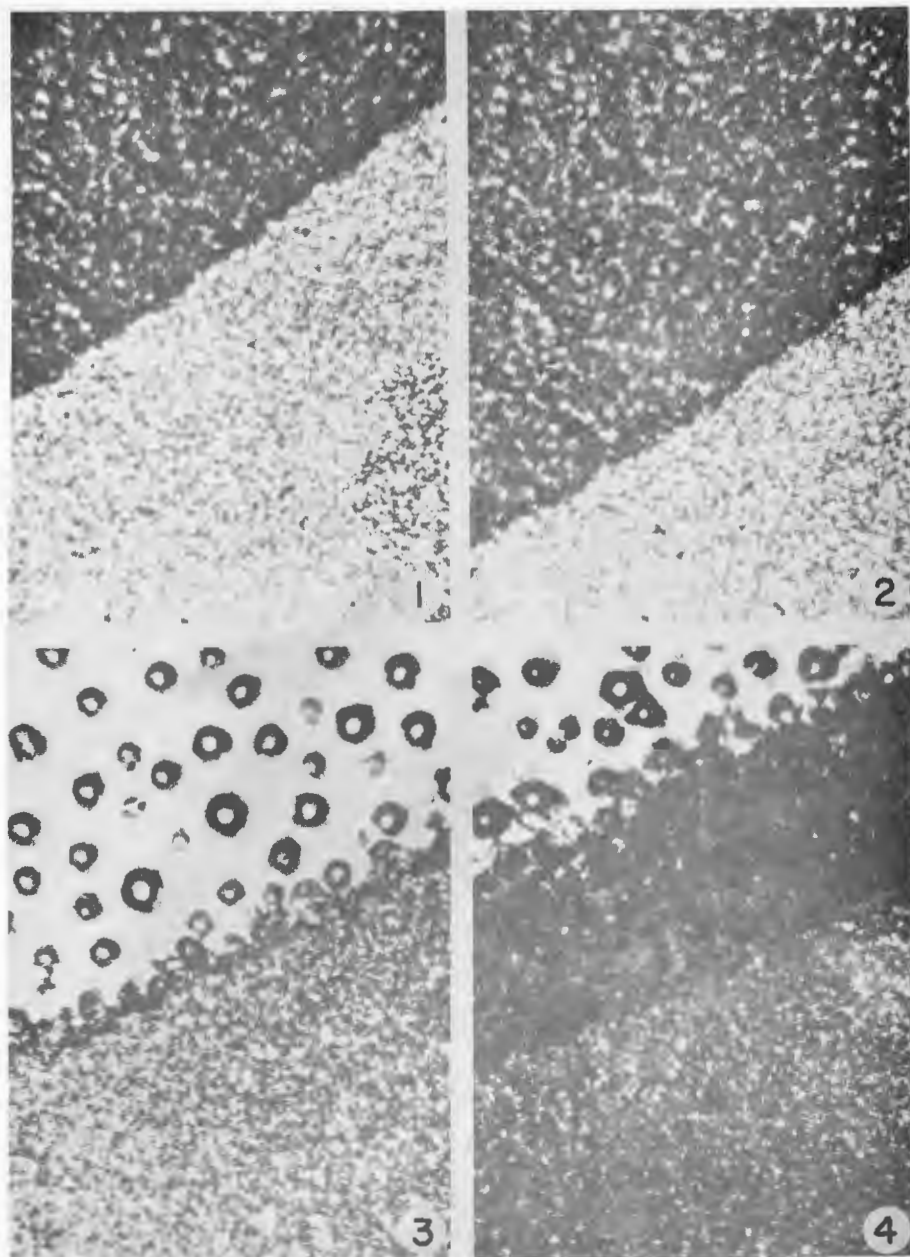


Plate 10. Single frames from a cinematographic record of the freeze-drying of banana "supernatant". See text for technique. Drying progresses, in each case, from upper left to lower right. — Photos. 1 and 2: sample temperature: -36° (freeze-drying progresses with "retention"). Photo. 3: sample temperature: -30° (freeze-drying proceeds with "collapse"). Photo. 4, upper part: sample temperature: -30° ; middle band: sample temperature: -36° . (When the temperature is lowered "collapse" ceases and freeze-drying progresses with "retention".) — Magnif.: ca. 300X.

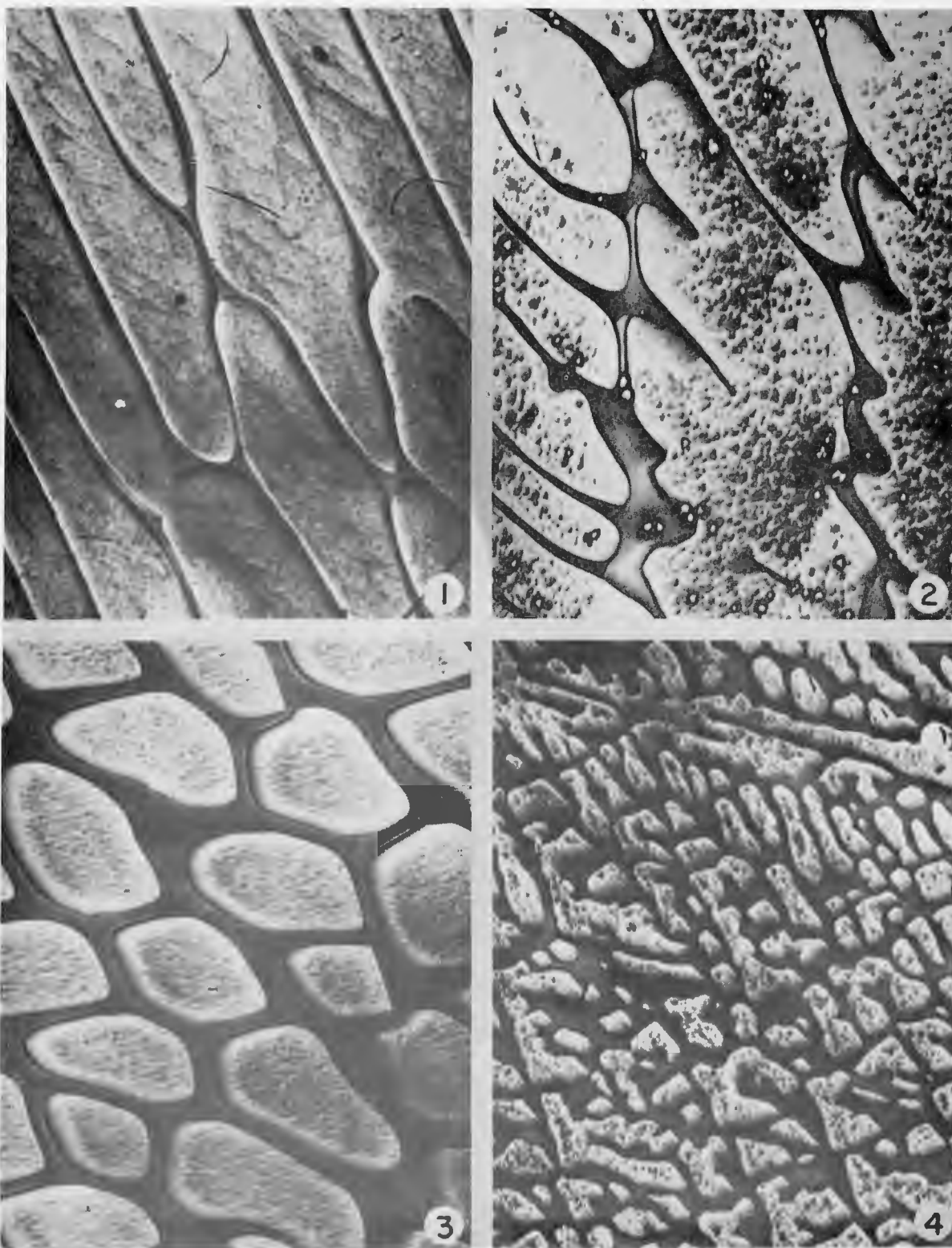


Plate 11. Electron micrographs showing the effect of solute concentration on the freezing pattern. Formvar replicas of surfaces created by cross fractur of sucrose solutions frozen in layers 1.2 mm. thick by abrupt immersion in isopentane at -140°C . — Concentration: Photo. 1: 10%; Photo. 2: 20%; Photo. 3: 30%; Photo. 4: 40%. — Magnif.: 5,700X.

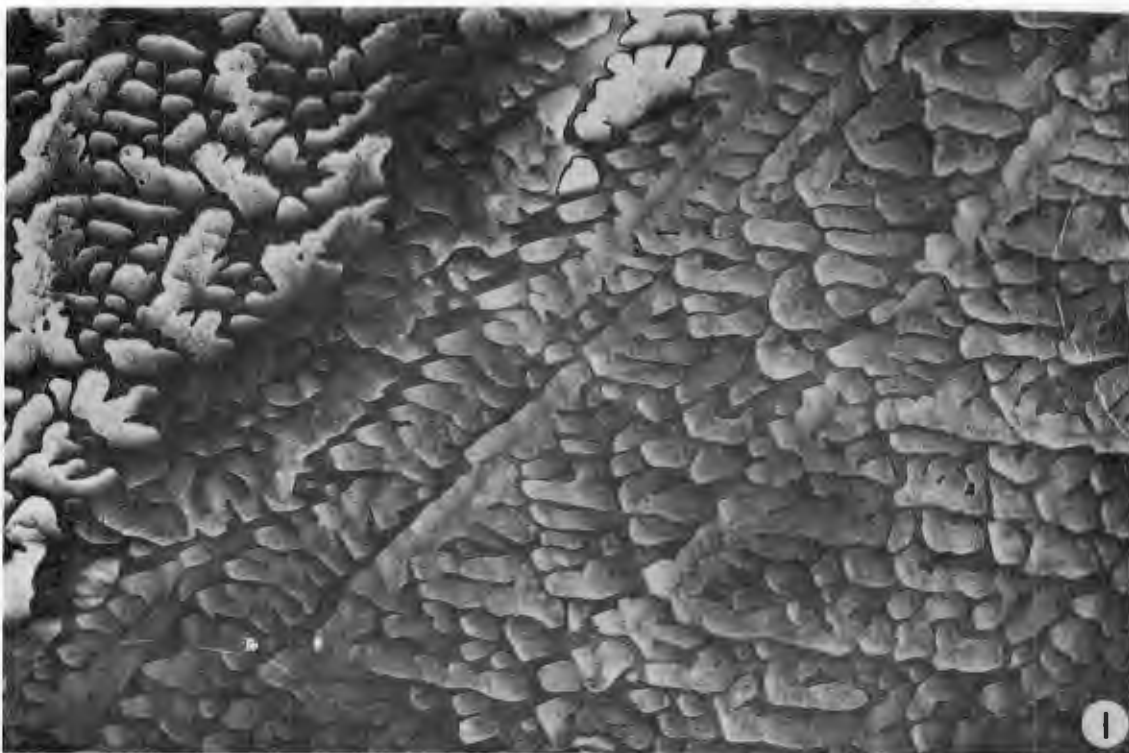


Plate 12. Electron micrographs of Formvar replicas of frozen sucrose solutions, illustrating the effect of rewarming in causing recrystallization. — Solute concentration: 35%. — Photo. 1: after freezing in isopentane at -140°C ; Photo. 2: after the same rapid freezing and a subsequent exposure to -30° for 20 min. — Sample thickness: 1.2 mm. — Magnif.: 5,700X.

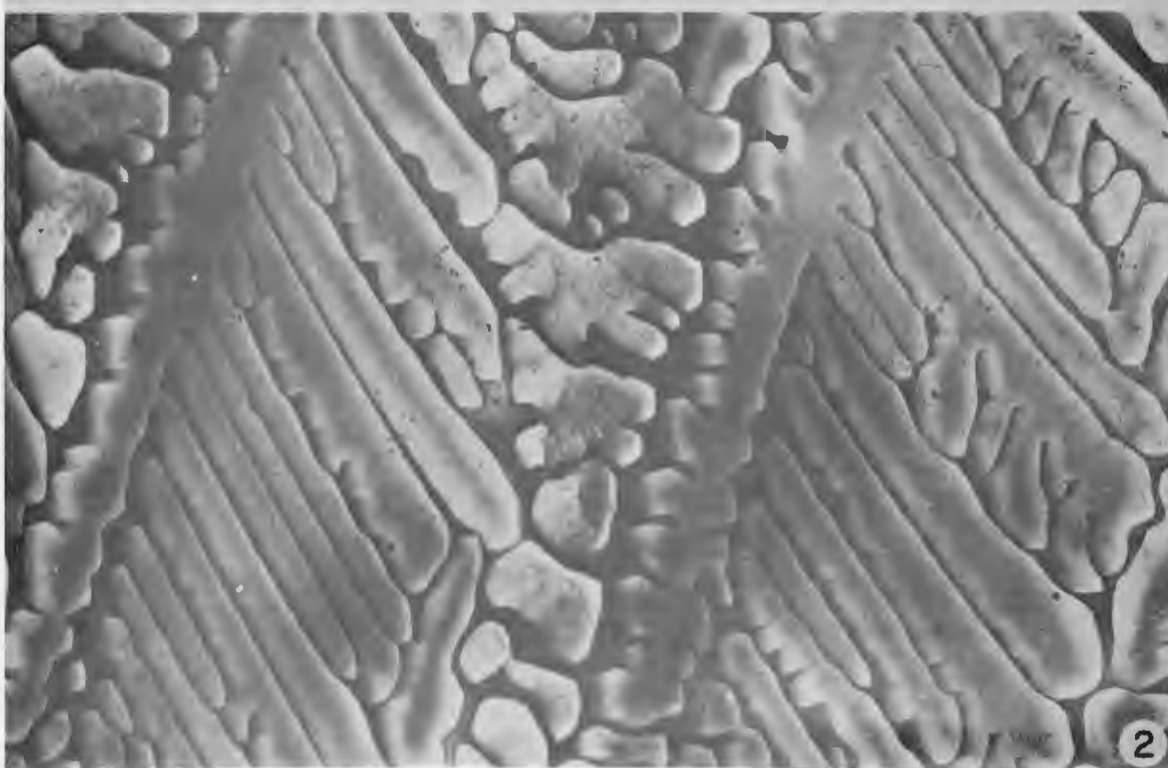
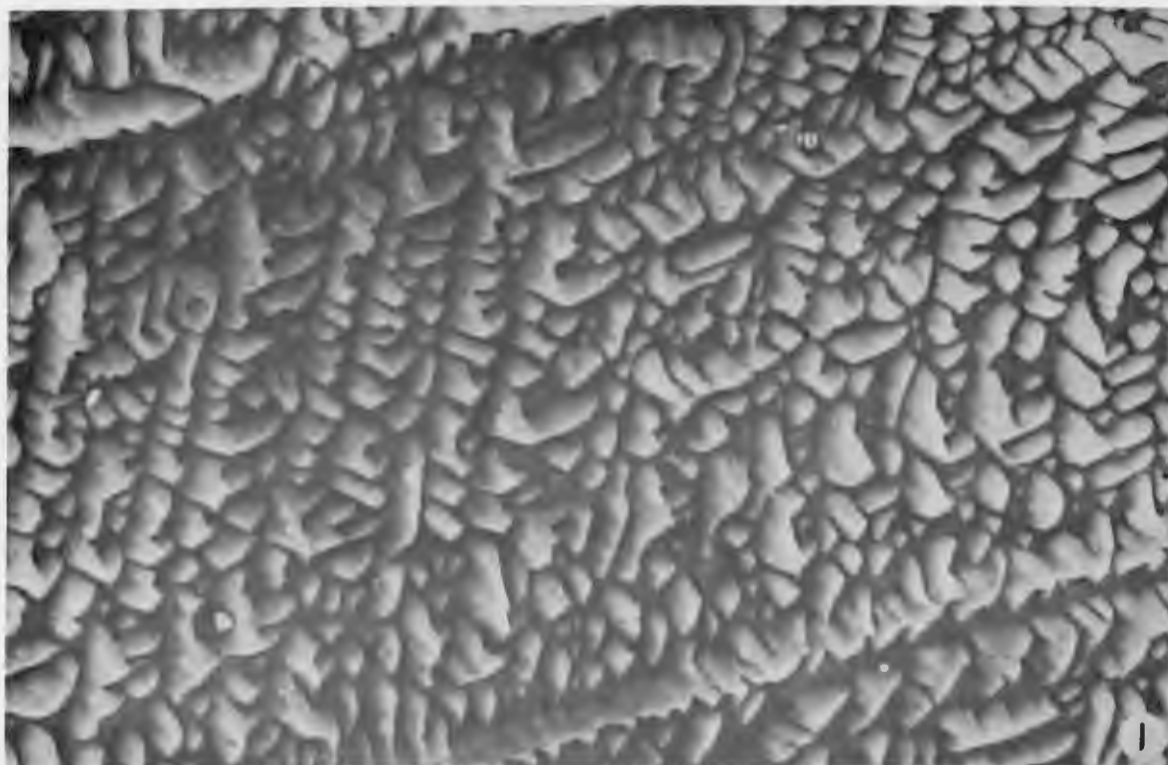


Plate 13. Electron micrographs exhibiting patterns which appear to depend on the relationship between the plane of fracture of the sample and the direction of growth of the ice dendrites. Formvar replicas from sucrose solutions frozen in isopentane at -140°C . — Concentration: Photo. 1: 35%; Photo. 2: 30%. — Magnif.: 5,700X.

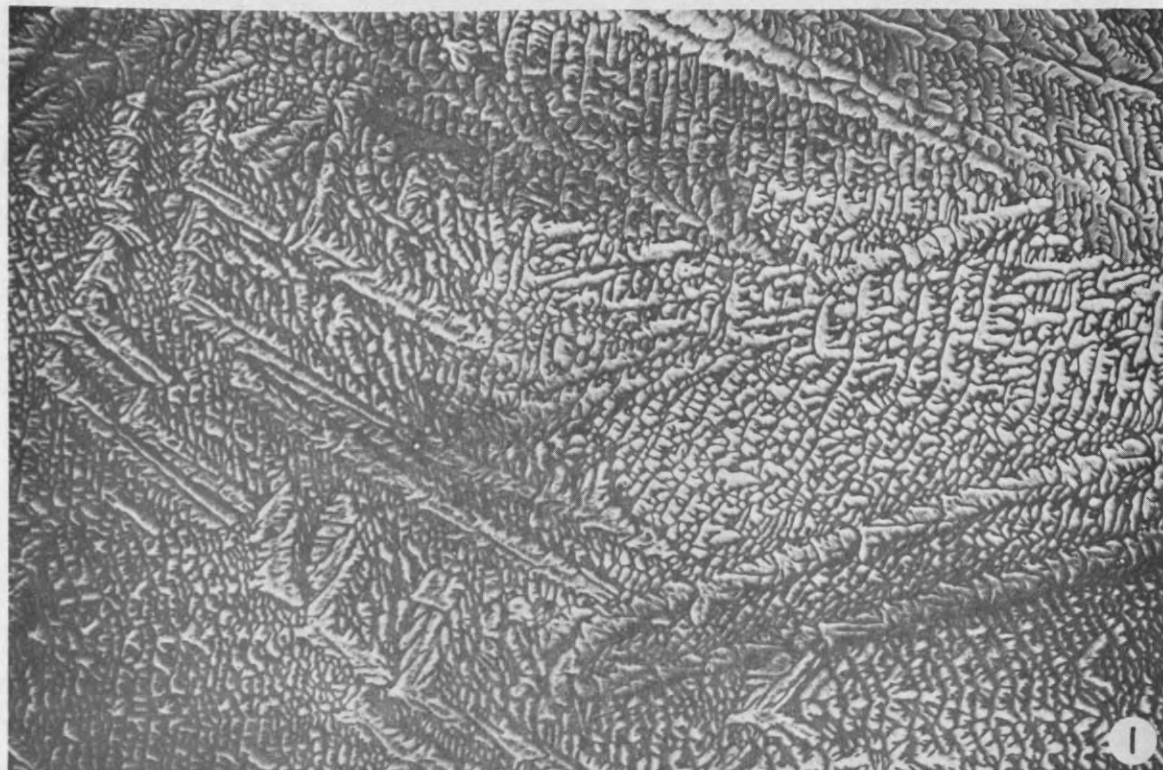


Plate 14. Electron micrographs of Formvar replicas of sucrose solutions illustrating the various extents to which dendrites with rectilinear branches arise after freezing by immersion in isopentane at -140°C . — Solute concentration: Photo. 1: 35%; Photo. 2: 30%. — Magnif.: 1,800X.

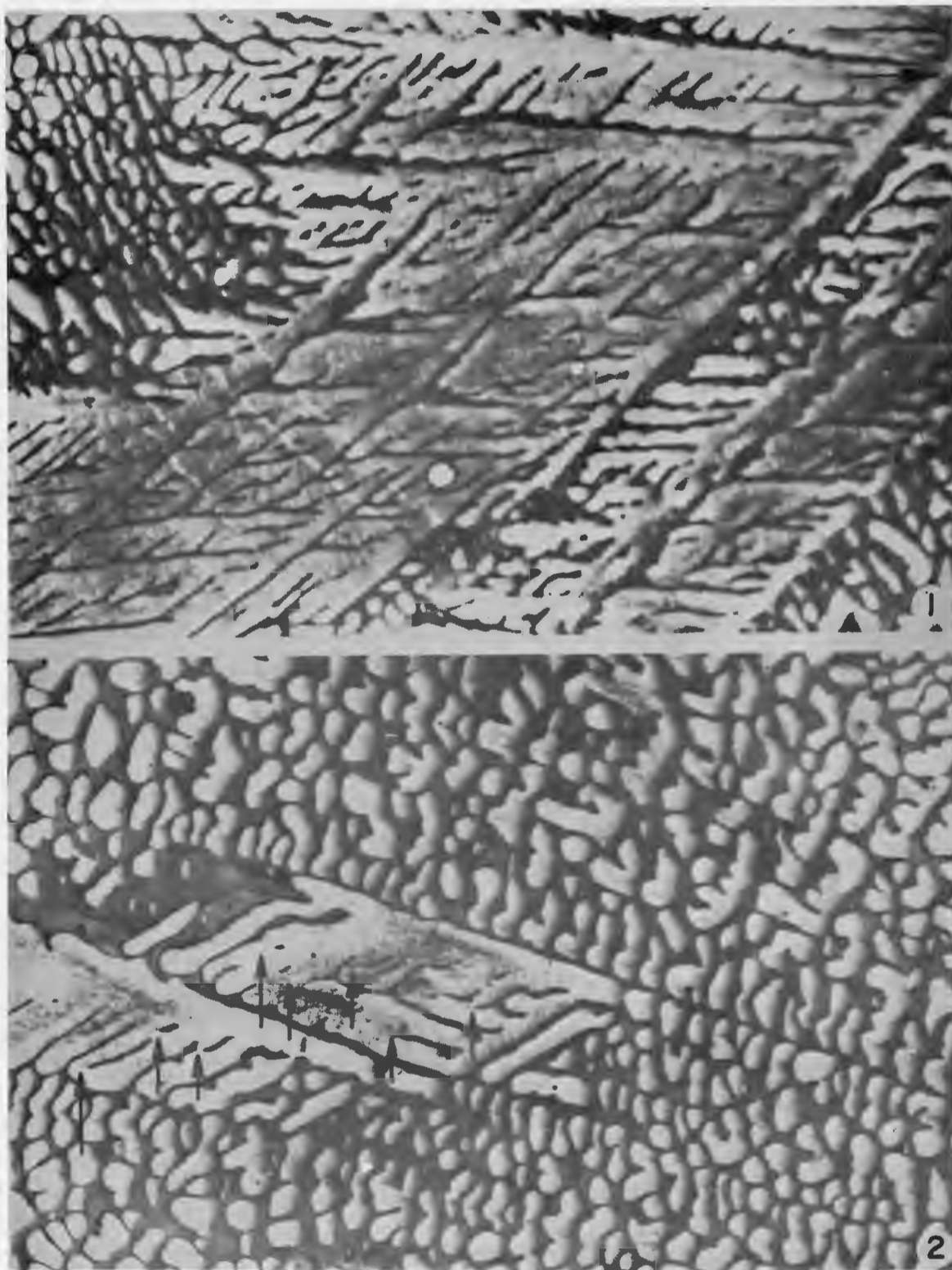


Plate 15. Electron micrographs showing some special features of dendritic structures which arose from rapid freezing of 35% sucrose solutions. — Photo. 1, at arrow: Main branch of a dendrite which would constitute a "bottleneck" during freeze-drying; Photo. 2, at arrows: Recrystallization of ice during freezing causing branches and subbranches to fuse and to form multiple ring structures. — Magnif.: 5,700X.

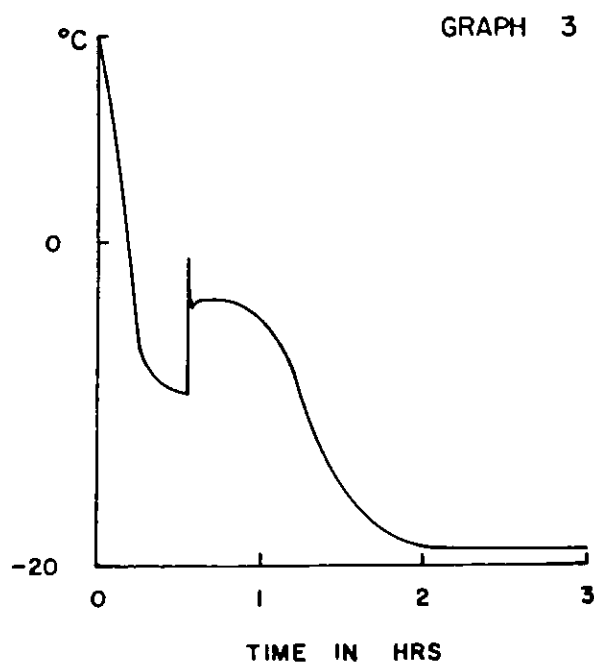
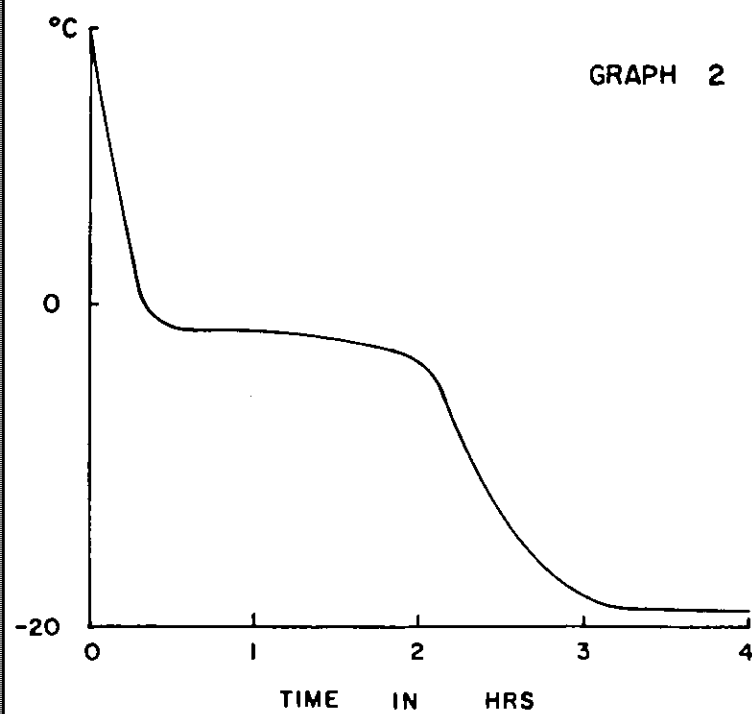
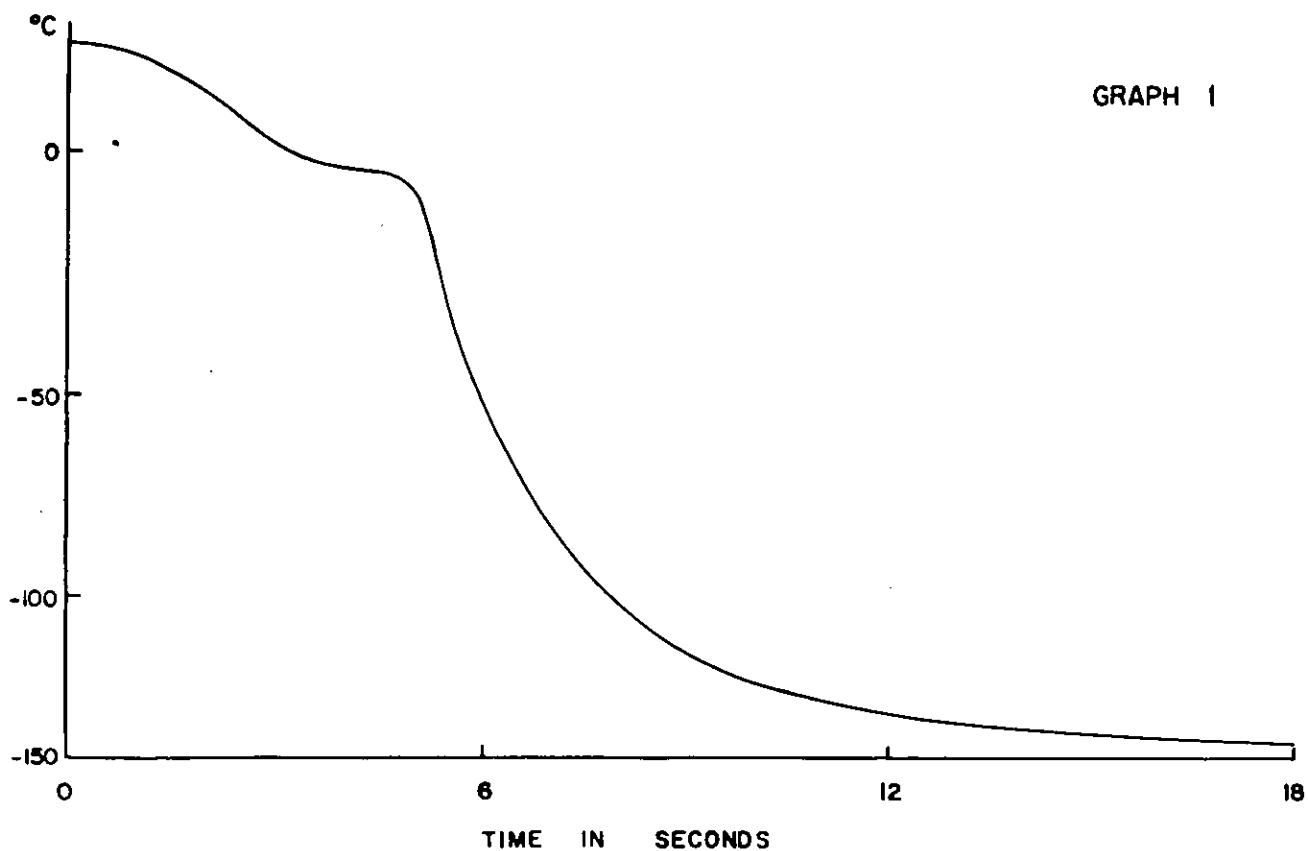


Fig. 1. Graph 1: Freezing curve of a piece of strawberry cooled rapidly by immersion in an isopentane bath at -150° . Graphs 2 and 3: Freezing curves of a piece of tomato (Graph 2) and of a pea (Graph 3) cooled slowly in air at -20° .

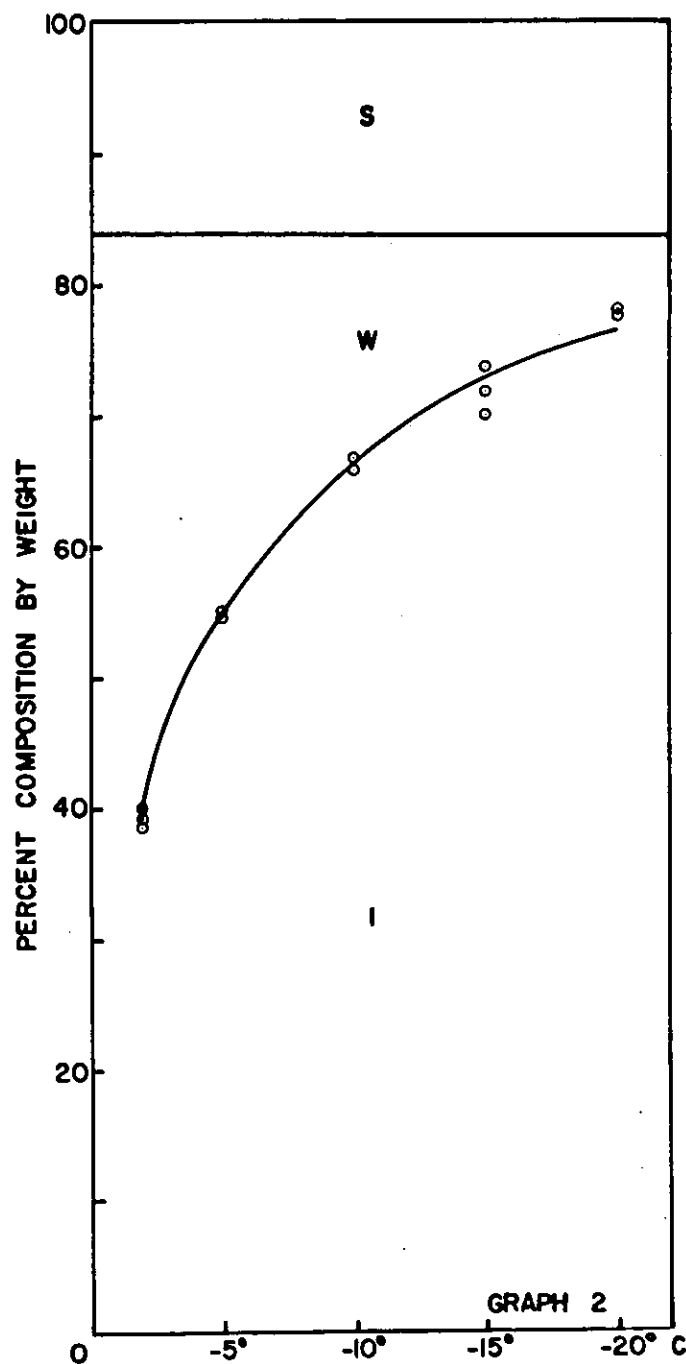
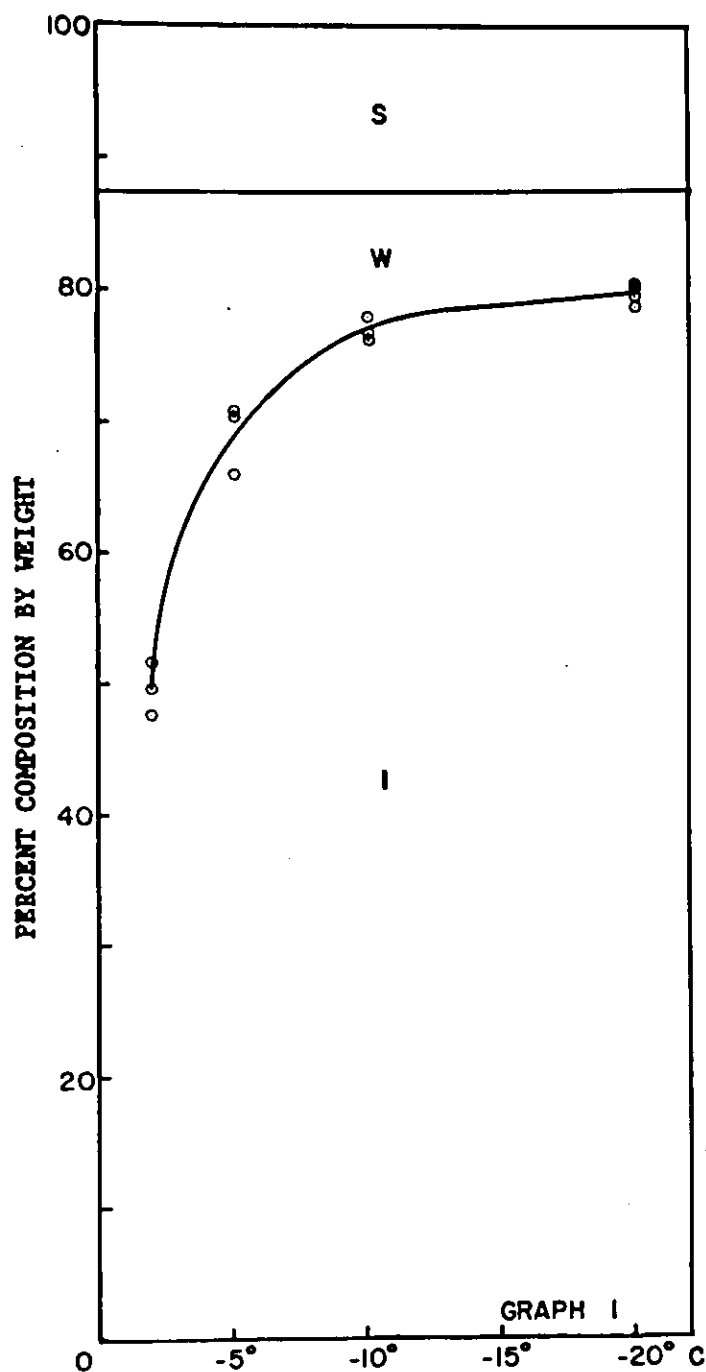


Fig. 2. Curves showing the relative proportions of ice (I), nonfrozen water (W) and solids (S) in peach (Graph 1) and in pineapple (Graph 2) frozen at the temperatures indicated in abscissa.

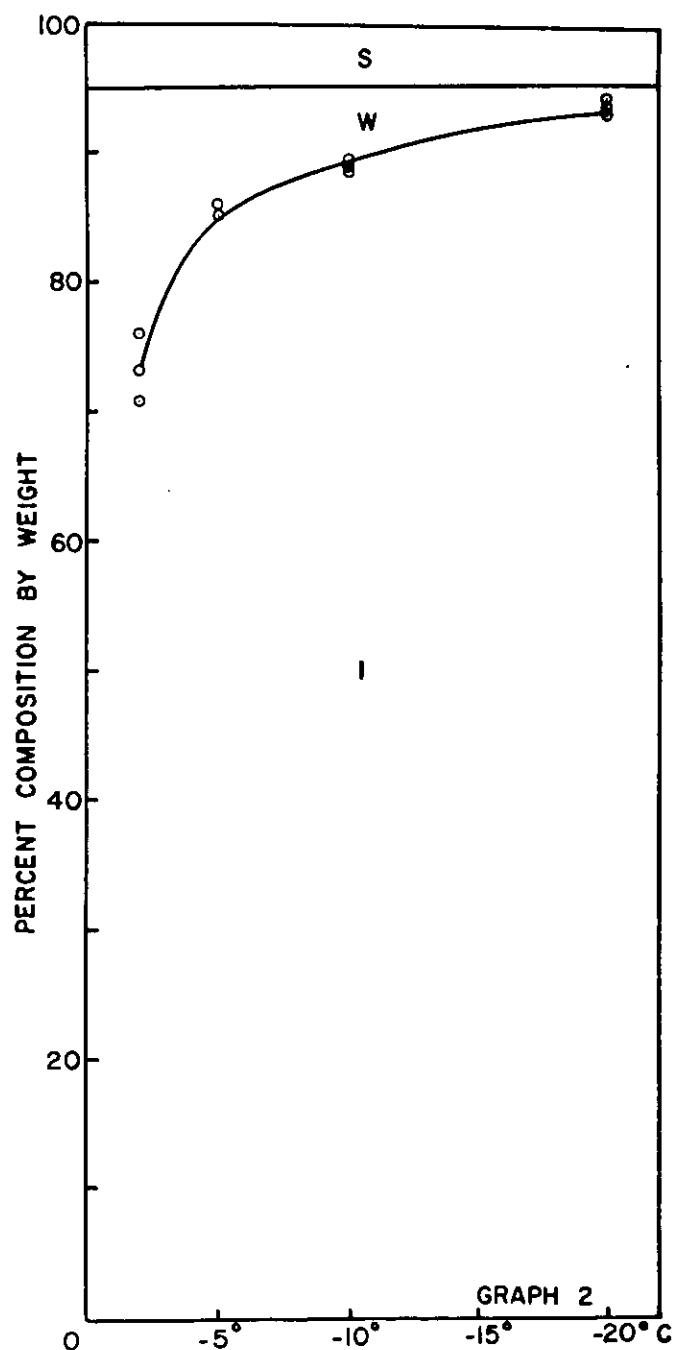
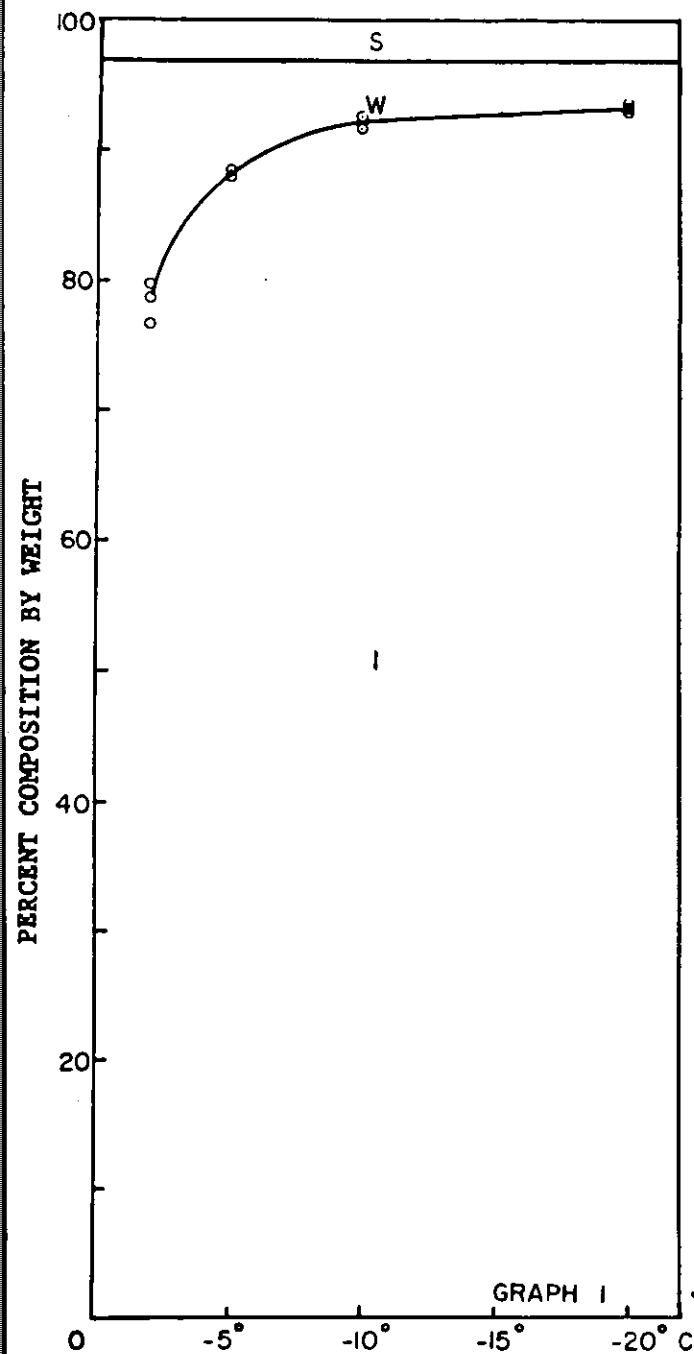


Fig. 3. Curves showing the relative proportions of ice (I), nonfrozen water (W) and solids (S) in lettuce (Graph 1) and in tomato (Graph 2) frozen at the temperatures indicated in abscissa.

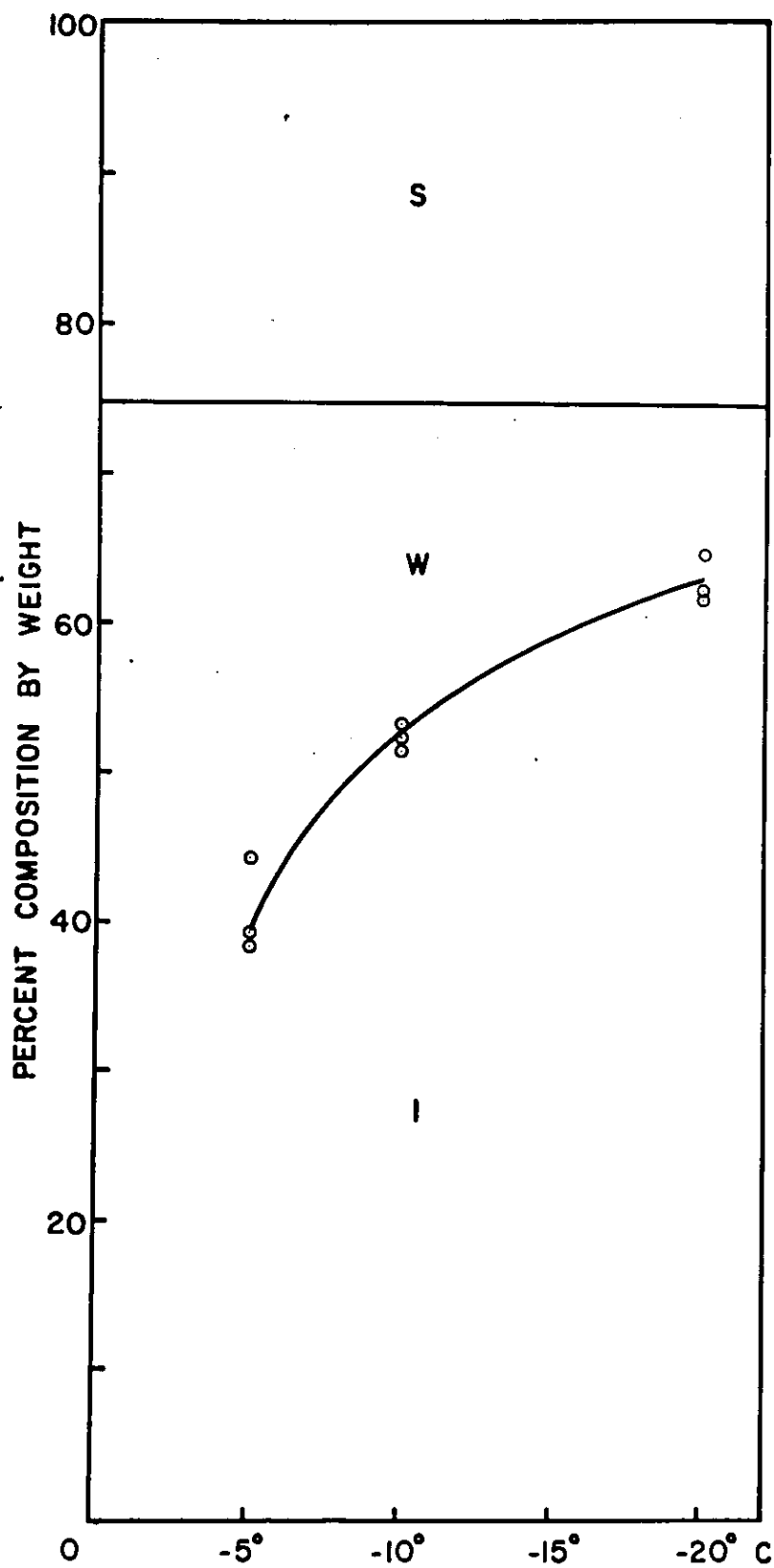


Fig. 4. Curve showing the relative proportions of ice (I), nonfrozen water (W) and solids (S) in banana frozen at the temperature indicated in abscissa.

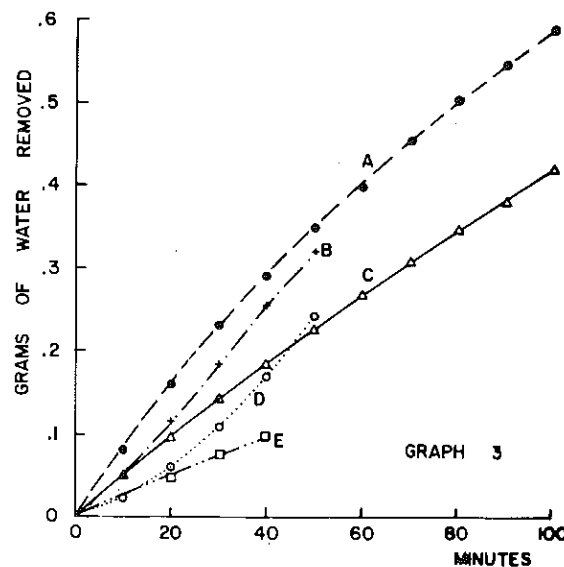
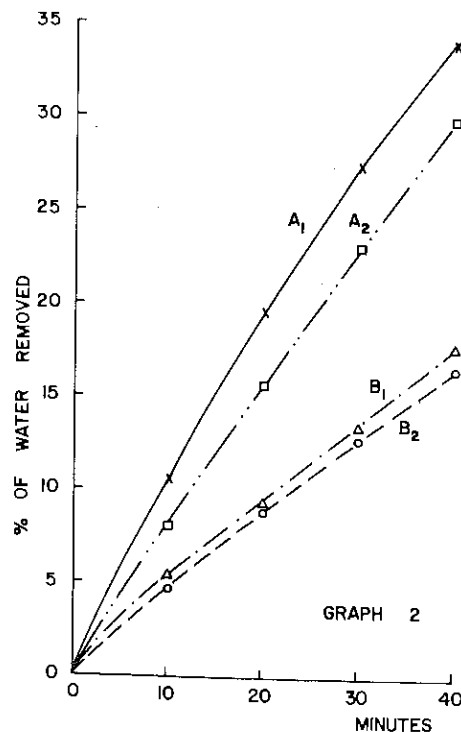
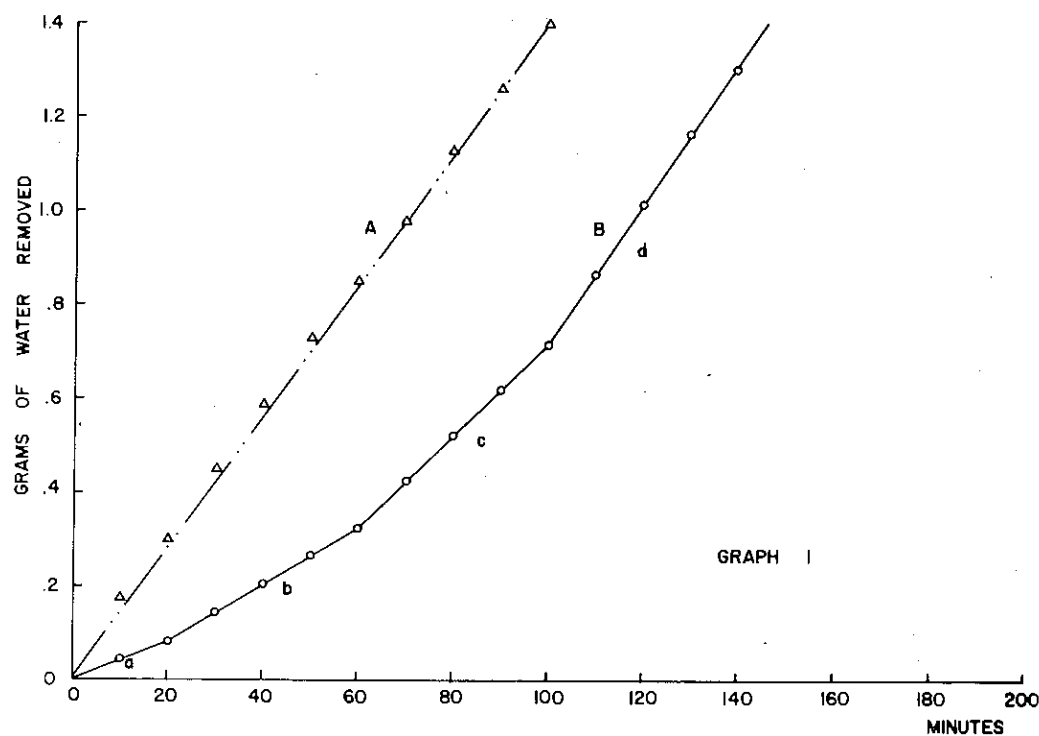


Fig. 5. Freeze-drying rates of banana tissue and its constituents. Graph 1: Rate of freeze-drying from one face of banana slices (frozen at -30°) as a function of the ambient temperature. — Curve A: Specimen freeze-dried continuously at -15° ; Curve B: Specimen freeze-dried consecutively at -30° , -25° , -20° and -15° (segments a, b, c and d, resp.). Graph 2: Comparative rates of freeze-drying, at -10° , of central and peripheral regions of banana slices. — Curves A₁ and A₂: Peripheral annulus and central disk, resp., of 1-mm.-thick slice frozen rapidly at -150° ; Curves B₁ and B₂: Annulus and disk, resp., of 1-cm.-thick slice frozen slowly at -30° . — Graph 3: Comparative freeze-drying rates of specimens, of identical size and shape, of intact banana tissue (C), banana paste (A), banana juice (E), and sugar solution (D), all frozen and freeze-dried at -30° ; Curve B: Sugar solution of same composition as used for E, but frozen at -78° .

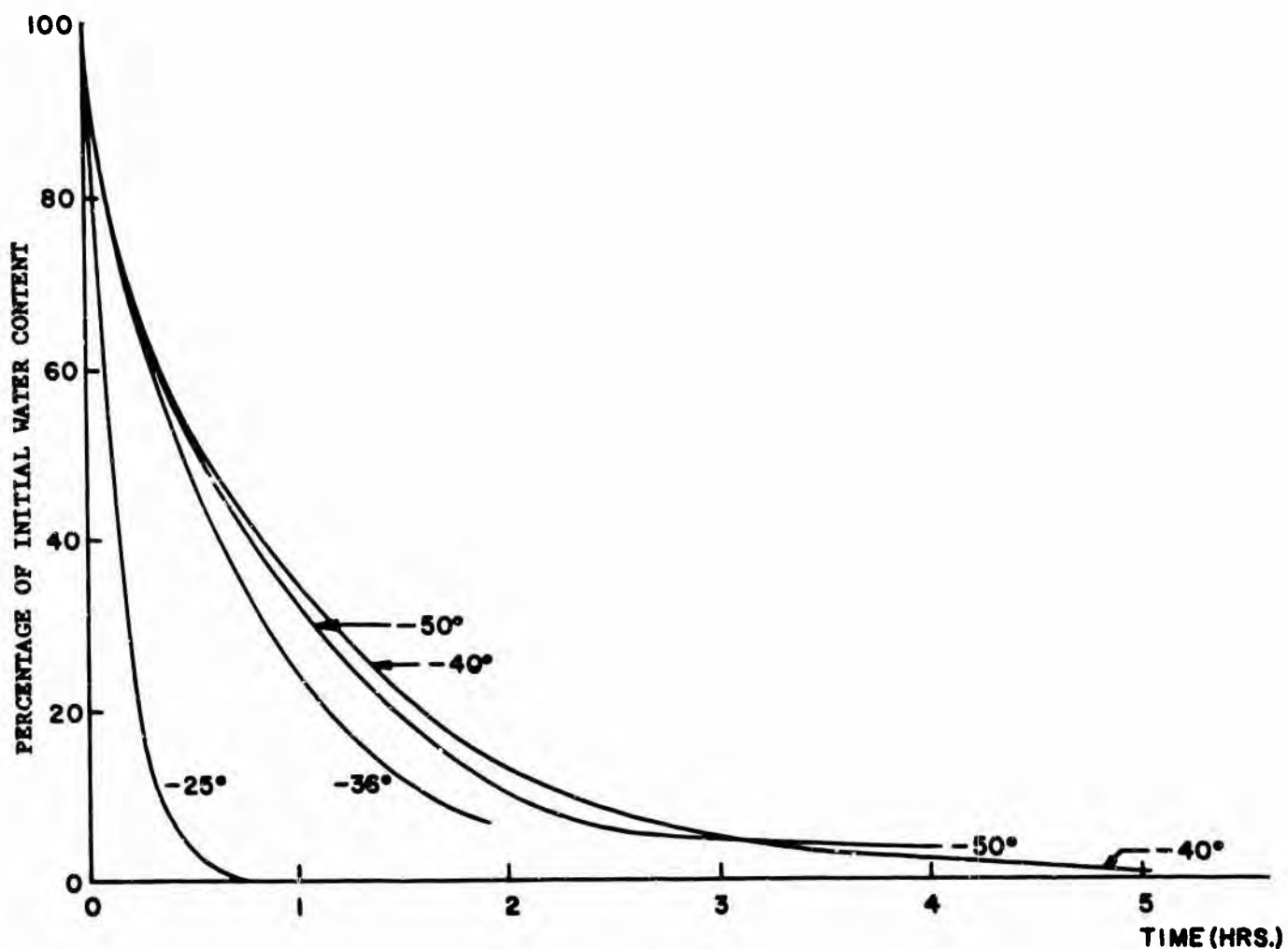


Fig. 6. Weight/time curves obtained during the freeze-drying of 25% sucrose solutions, rapidly frozen. The temperature of the subliming interface was maintained at the stated value throughout the determination.

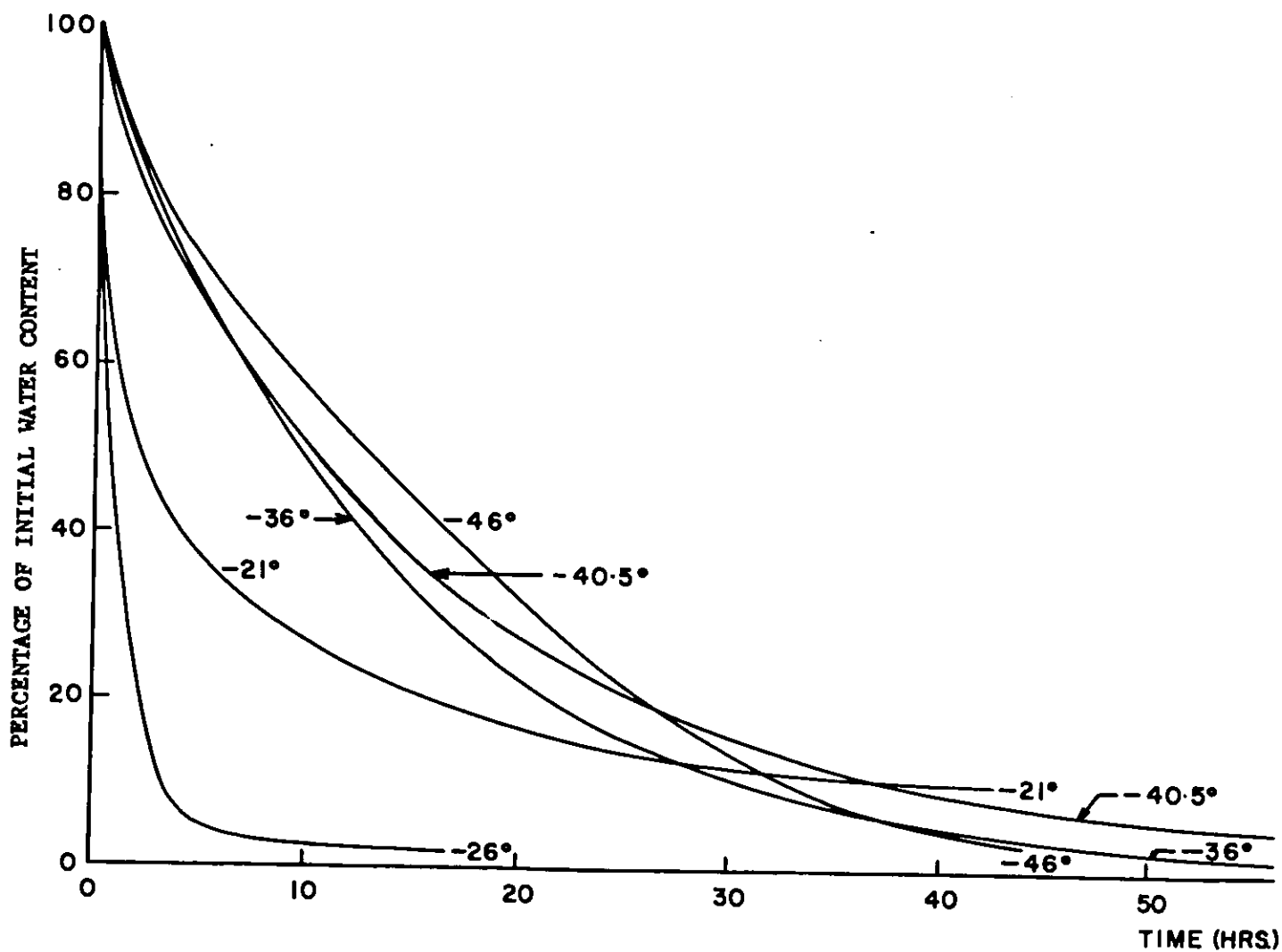


Fig. 7. Weight/time curves obtained during the freeze-drying of 36% sucrose solutions, rapidly frozen. The temperature of the subliming interface was maintained at the stated value throughout the determination.

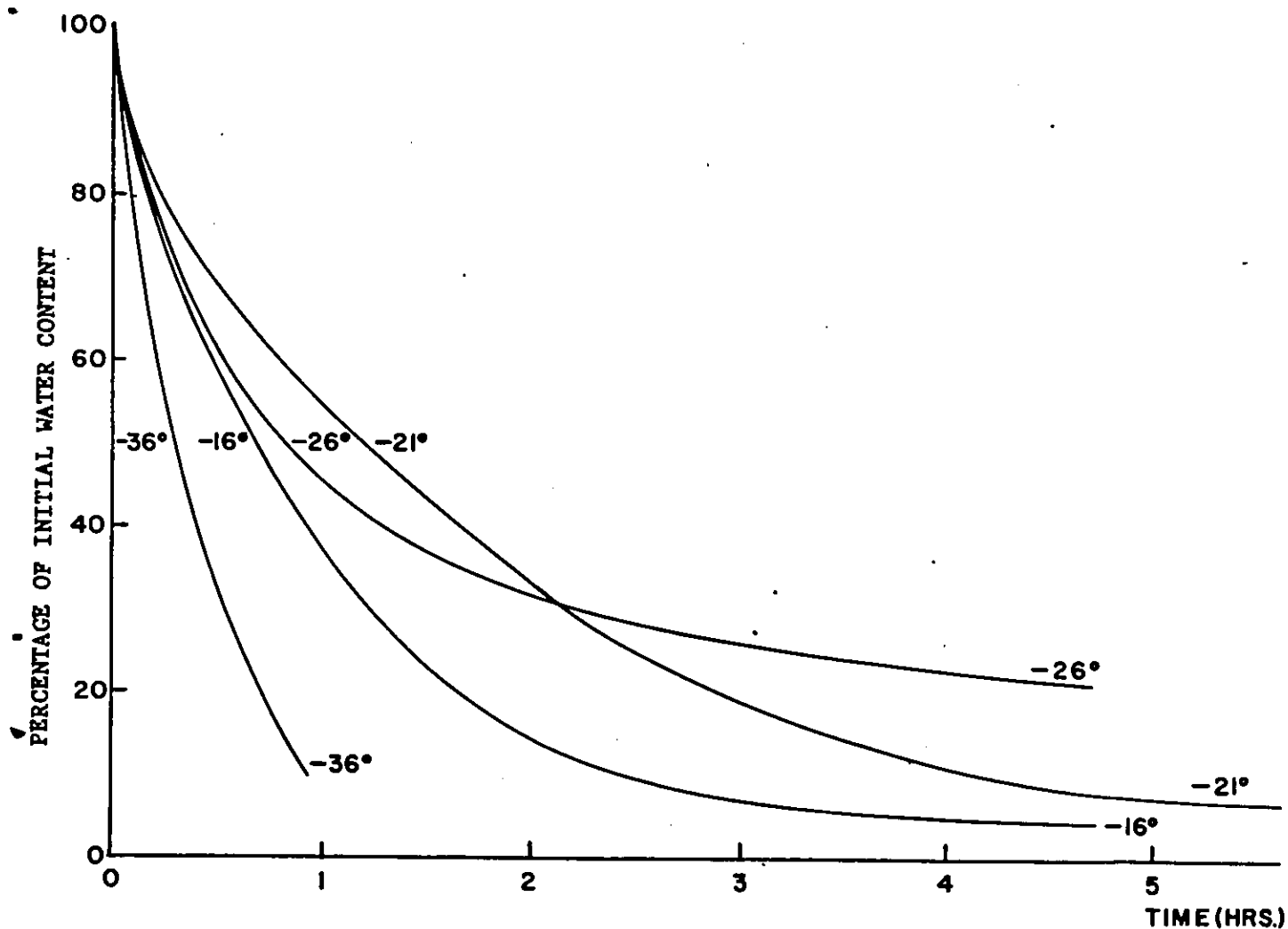


Fig. 8. Weight/time curves obtained during the freeze-drying of rapidly frozen banana "supernatant" fluid. The temperature of the subliming interface was maintained at the stated value throughout the determination. — Note that freeze-drying of this material proceeds much more rapidly at -36° than at -26° .

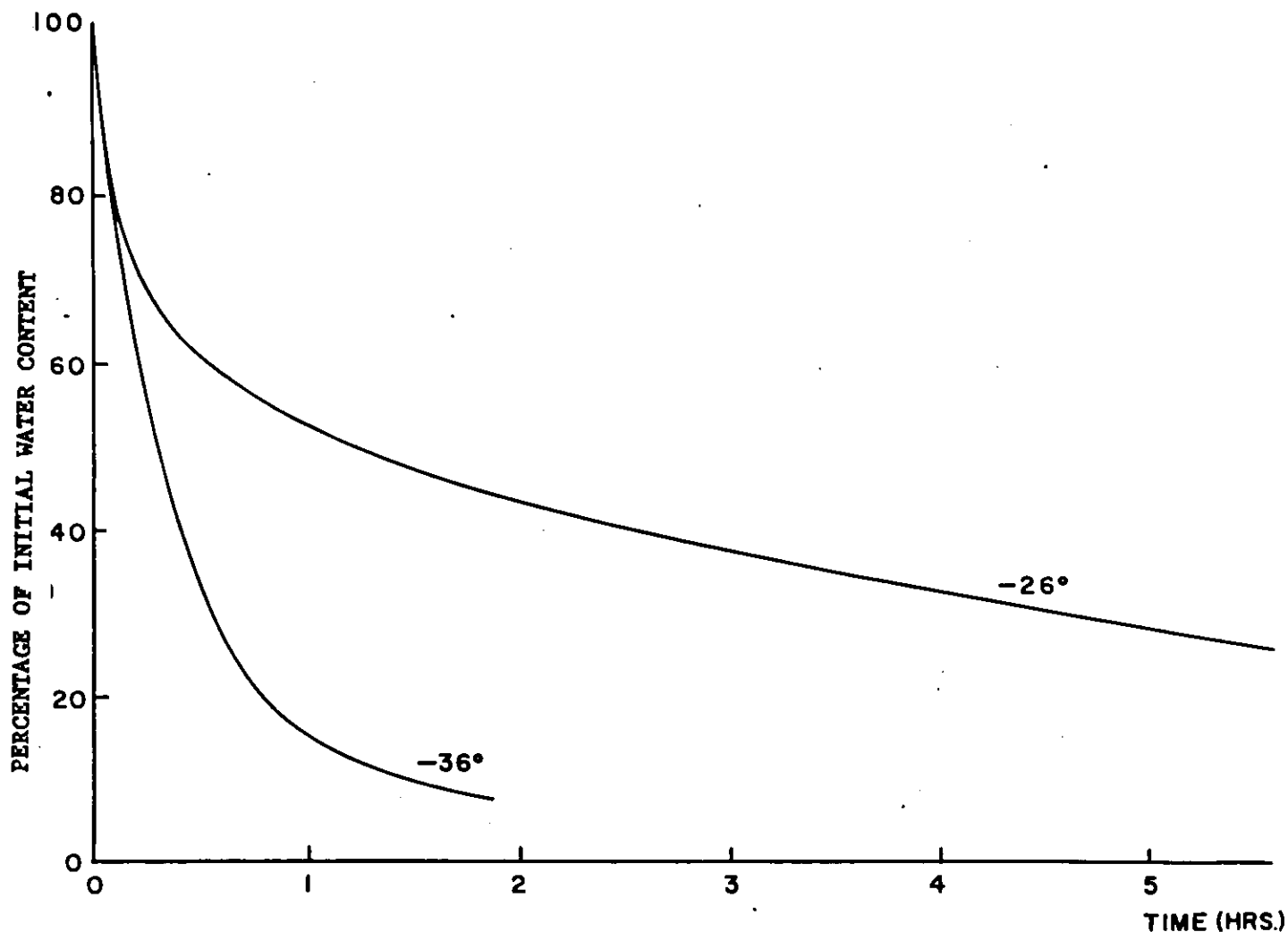


Fig. 9. Weight/time curves obtained in experiments conducted to test the reproducibility of the freeze-drying behavior of the "supernatant" at -36° and at -26° shown in Fig. 8. The results confirmed the previous finding that the freeze-drying rate is higher at the lower freeze-drying temperature.

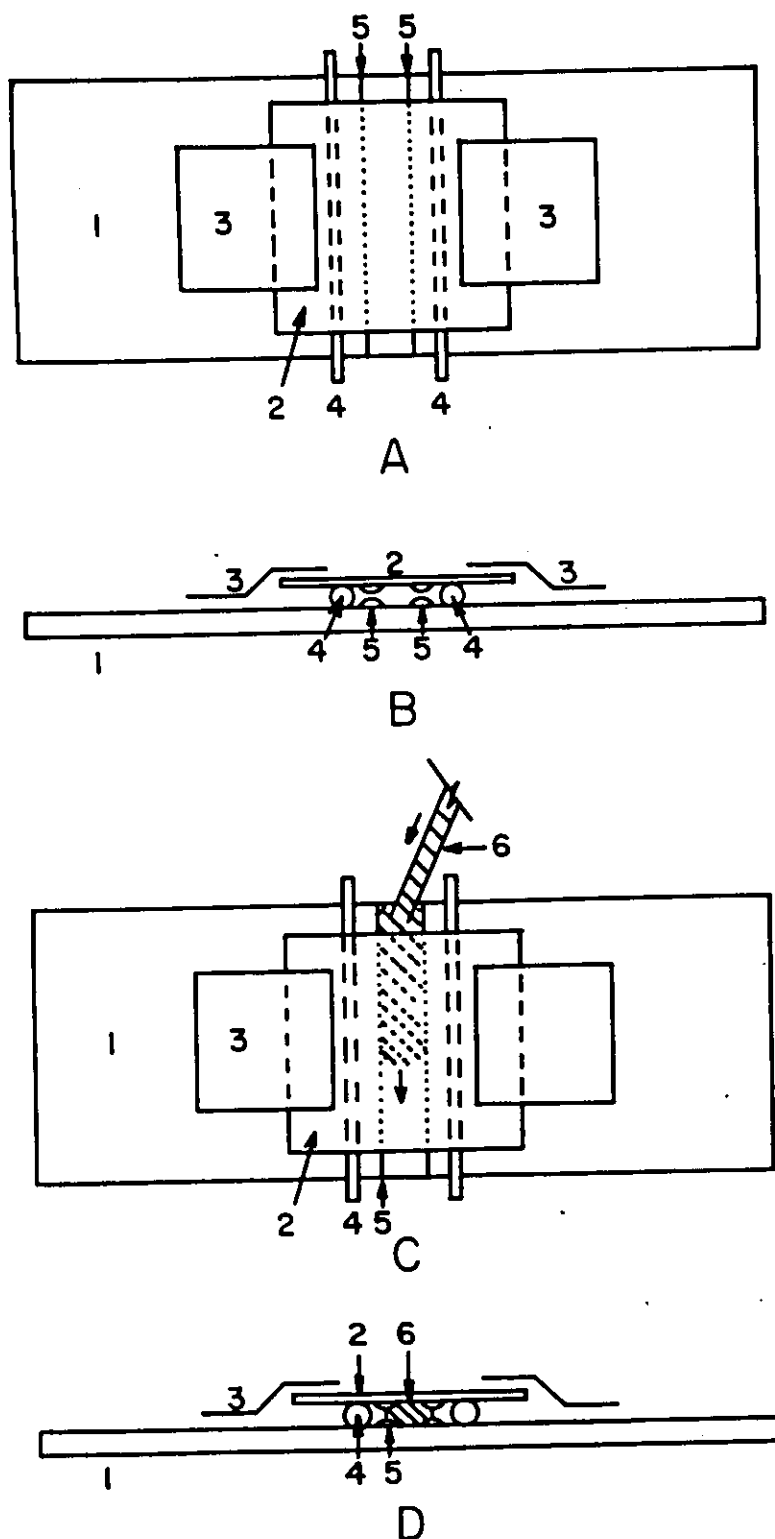


Fig. 10. Diagrams illustrating the method devised for the microscopic study of rehydration. A. Empty sample holder (plan view). B. Empty holder (elevation). C. Method of filling the holder (plan view). D. Holder with sample solution (elevation). 1: microscope slide; 2: cover slip; 3: masking tape; 4: wire spacer; 5: rubber cement; 6: sample solution. (Drawing not to scale.)

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<p>Investigations were made on the mode of invasion of fruit and vegetable tissue by ice and the changes produced by freezing and thawing and freeze-drying of the tissues as well as rehydration evaluations.</p> <p>The diversity of the structure and composition of the fruits and vegetables investigated required the use of different methods of preparation and observation. In general three methods of exploring the effects of freezing and freeze-drying were used. (1) Direct observation of thin sections of fresh tissue under a cryomicroscope during freezing and thawing. (2) Observations of material fixed and stained after it had been freeze-dried. (3) Observations of material sectioned while in the frozen state or after freeze-drying.</p> <p>Observations on the rehydration of thin tissue samples in a specially designed microscope demonstrated the dependence of the rehydration behavior on the structural features of the freeze-dried system.</p>			

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